High-throughput transformation pipeline for a Brazilian *japonica* rice with bar gene selection

B. Dedicova · C. Bermudez · M. Prias · E. Zuniga · C. Brondani

Received: 24 March 2014 / Accepted: 27 November 2014 / Published online: 7 December 2014
© The Author(s) 2014. This article is published with open access at Springerlink.com

Abstract The goal of this work was to establish a transformation pipeline for upland Curinga rice (*Oryza sativa* L. *ssp.* *japonica*) with bar gene selection employing bialaphos and phosphinothricin as selection agents. The following genes of interest: *AtNCED3*, *Lsi1*, *GLU2*, *LEW2*, *PLD-alpha*, *DA1*, *TOR*, *AVP1*, and *Rubisco* were cloned into the binary vector p7i2x-Ubi and were transferred into *Agrobacterium* strain EHA 105. Embryogenic calli derived from the mature embryos were transformed, and transgenic cells and shoots were selected on the medium supplemented with bialaphos or phosphinothricin (PPT) using a stepwise selection scheme. Molecular analyses were established using polymerase chain reaction and Southern blot for the bar gene and the NOS terminator. Overall, 273 putative transgenic plants were analyzed by Southern blot with 134 events identified. In total, 77 events had a single copy of the transgene integrated in the plant genome while 29 events had two copies. We tested backbone integration in 101 transgenic plants from all constructs and found 60 transgenic plants having no additional sequence integrated in the plant genome. The bar gene activity was evaluated by the chlorophenol red test and the leaf painting test using phosphinothricin with several transgenic plants. The majority of T0 plants carrying the single copy of transgene produced T1 seeds in the screen house.

Keywords *Oryza sativa* L · Upland rice · *Agrobacterium*-mediated transformation · Bar selection · Backbone integration

Handling Editor: Burkhard Becker

B. Dedicova (✉) · C. Bermudez · M. Prias · E. Zuniga
International Center for Tropical Agriculture A.A. 6713,
Cali, Colombia
e-mail: beatadedicova@hotmail.com

C. Brondani
Embrapa Arroz e Feijão, Rodovia GO-462, Km 12 75.375-00,
Santo Antônio de Goiás, Goiás, Brazil

Introduction

Rice equates to life for thousands of millions people in Asia alone where more than 2000 million people obtain 60 to 70 % of their calories from rice and its products (FAO 2013). In Latin America, rice is the most important food grain providing 27 % of daily calorie intake overall, ranging from 8 % in Central America to 47 % in the Caribbean region (FAOSTAT 2012). Upland rice, known as aerobic rice, is grown in South America, Africa, and Asia (Fageria 2002). In the central part of Brazil (Cerrados), upland rice plays an important role in the cropping system whereby it is first grown after clearing land for pasture (Fageria 2010). The main limiting factors for adopting high-yielding rice varieties is drought and access to nitrogen in drought-prone rainfed rice environments (Fageria 2009). These two traits, drought tolerance and nitrogen-use efficiency, have been of high interest in past experimental research (Campbell et al. 1995; Umezawa et al. 2006; Serraj et al. 2008) and have been reviewed several times in recent years (Hadiarto and Tran L-S 2011; Lawlor 2013). Dozens of genes with different functions and modes of action have been identified (Shinozaki and Yamaguchi-Shinozaki 2007; Yang et al. 2010; Jeong et al. 2013) with several going through confined field testing (Deikman et al. 2011; Gaudin et al. 2013).

This study focused on following genes linked to plant stress resistance, plant growth, and yield: The *AtNCED3* encodes the key enzyme in the abscisic acid (ABA) biosynthesis via overexpression of 9-cis-epoxycarotenoid dioxygenase in Arabidopsis (Iuchi et al. 2001). The *Lsi1* gene (Low silicon rice 1) encodes the Si transporter from the aquaporin gene family, is expressed in rice roots (Ma et al. 2006), and is downregulated during dehydration stress by ABA (Yamaji and Ma 2007). The *AtCESA8/IRX1* gene from the Lew2 Arabidopsis mutants (Chen et al. 2005) is one of ten genes essential for the cellulose synthase complex in the secondary cell walls (Taylor 2008). The phospholipase *Dα1* (*PLD α1*) gene is involved in the stress response through

stomata closure directed by the ABA effect (Mishra et al. 2006; Uraji et al. 2012). Expression of the *AtTOR* kinase (target of rapamycin) influences seed and plant growth and controls resistance to osmotic stress (Deprost et al. 2007). Disruption of the *TOR* activity can lead to premature arrest of endosperm and embryo development (Menand et al. 2002). Another gene controlling seed and organ size is the *DAI* gene, which encodes the ubiquitin receptor that controls the cell proliferation period (Li et al. 2008) and final seed and organ size as well as increases plant biomass. *AVP1* encodes a vacuolar pyrophosphatase which functions as a proton pump in the vacuolar membrane and, in transgenic *Arabidopsis* plants, expression of this gene can increase the vacuolar proton gradient resulting in elevated solute accumulation and water retention (Gaxiola et al. 2001). If this gene is overexpressed in cotton, transgenic plants display improved tolerance to drought and salt stress (Pasapula et al. 2011). The major role of the *AtGLU2* gene is nitrogen assimilation in plant roots (Coschigano et al. 1998; Lancien et al. 2002). A positive correlation between nitrogen content and photosynthetic capacity through RuBisCO (ribulose-1, 5-bisphosphate carboxylase/oxygenase) is well-documented in higher plants (Evans 1989). In rice, the multiple gene family *rbcS* consists of five genes whose expression is enhanced by increased nitrogen (Suzuki et al. 2007; Miyazaki et al. 2013). The relationship between the *rbcS* and *rbcL* genes leads to leaf senescence and nitrogen influx (Imai et al. 2008).

The bar gene-based selection system produced numerous herbicide-resistant biotech crops, e.g., oilseed rape (De Block et al. 1989; Kopertekh et al. 2009), lettuce (McCabe et al. 1999), soybean (Zeng et al. 2004), mungbean (Sonia et al. 2007), carrots (Jayaraj et al. 2008), sweet potato (Zang et al. 2009), sugarcane (Joyce et al. 2010), cassava (Koehorst-van Putten et al. 2012), and tomato (Khuong et al. 2013).

The bar gene selection was also successfully used for conifers, *Pinus radiata* (Charity et al. 2005), orchids (Knapp et al. 2000), and flower species (Kamo and Young 2009; Chen et al. 2010b).

Among the monocotyledonous plants, e.g., wheat (Weeks et al. 1993; Wu et al. 2008), maize (Zhang et al. 1996), barley (Wu et al. 1998), oat (Kuai et al. 2001), Bermuda grass (Hu et al. 2005), tropical maize (Valdez-Ortiz et al. 2007), and ryegrass (Patel et al. 2013) were reported. The rice transformation protocols using *Agrobacterium tumefaciens* technology were widely established for several genotypes with either hygromycin (Hiei et al. 1994) or phosphinothricin (PPT) (Toki et al. 1992) selection and continue to represent two dominate selection systems currently used for this crop (Bajaj and Mohanty 2005; Chen et al. 2010a; Twyman et al. 2002).

In this study, we used ten different genes of interest as described above, which are involved in abiotic stresses mainly drought tolerance, including the ABA and nitrogen signaling

pathway and plant growth. Our goal was to establish an *Agrobacterium*-mediated transformation protocol for Curinga, a commercial upland rice variety from Brazil in combination with bar gene selection using a PPT and bialaphos stepwise selection scheme.

Materials and methods

Plant material

The donor Curinga plants for the mature embryo/seed production were grown in the screen house at temperature 32–35 °C/20 °C (day/night) with a 12/12 photoperiod. Surface sterilized mature embryos were cultured scutellum side up for 2 weeks on Chu (Chu 1978) callus induction medium (Table 1). After 15 days, induced embryogenic calli were subcultured to the same medium and cultures proliferated for another 15 days in the same culture conditions at 24–26 °C in darkness. Well-developed embryogenic calli were subcultured 3 days prior to the transformation experiments to the same Chu callus induction medium supplemented additionally with 100 µM acetosyringone and were maintained in darkness at 24–26 °C. For composition of all media used in this study, see Table 1.

PPT and bialaphos tissue sensitivity test

Different concentrations of PPT or bialaphos in Chu medium and MS medium (Murashige and Skoog 1962) were tested for different stages of rice culture development: embryogenic calli proliferation on Chu medium supplemented with 3, 5, and 7 mg/l and MS medium for the shoots' induction, proliferation, and rooting supplemented with 1, 3 and, 5 mg/l. The lethal dose (LD) 50 and 100 were determined for different developmental stages, and those concentrations were used in a stepwise selection scheme for transformation experiments to select transgenic cells and induced shoots allowing them to form roots in the presence of the selection agent.

Constructs, transformation, and plant selection

The binary vector p7i2xU was used in combination with the following genes of interest: *AtNCED3*, *AtGLU2*, *Lsi1*, *LEW2*, *PLD-alpha*, *DAI*, *TOR*, *AVP1*, and two *Rubisco* genes (provided by Dr. Hermann Schmidt from DNA Cloning Service, Germany) were driven by maize Ubi-1 promoter and selection marker gene. The bar gene was driven by doubled CaMV 35 S promoter in all constructs (Fig. 1). Ten constructs were individually transferred into *A. tumefaciens* strain EHA 105 by electroporation (Wen-jun and Forde 1989).

Table 1 Media used

Medium	Abbreviation	Composition
Callus induction	Chu-Ind.	Chu medium (macro, micro elements, vitamins), 500 mg/l L-proline, 500 mg/l L-glutamine, 300 mg/l casein enzymatic hydrolyzate, 100 mg/l myo-inositol, 30 g/l maltose, 2.5 mg/l 2,4-D, 3 g/l gelrite, pH 5.8
Pre-culture	Chu + AS	as Chu-Ind. plus 100 μ M acetosyringone, pH 5.8
YEP		5 g/l yeast extract, 10 g/l peptone, 5 g/l NaCl, 15 g/l bacteriological agar, pH 7
Chu-Infection	Chu-Inf.	Chu medium (macro, micro elements, vitamins), 2 mg/l 2, 4-D, 1 g/l casamino acids, 15 g/l maltose, 15 g/l glucose, pH 5.2
Co-culture	Chu + AS	as Chu-Ind. plus 100 μ M acetosyringone, pH 5.8
Washing	Chu-W	Chu medium (macro, micro elements, vitamins), 500 mg/l cefotaxime, pH 5.8
Selection	Chu S1, S2	Chu-Ind. plus 3, 5 mg/l PPT or bialaphos, 250 mg/l cefotaxime, pH 5.8
Shoots induction	MS Ind.	MS medium (macro, micro elements, vitamins), 1 mg/l NAA 4 mg/l kinetin, 250 mg/l cefotaxime, 3 mg/l PPT or bialaphos, pH 5.8,
Shoots rooting	MS-R	MS medium (macro, micro elements, vitamins) hormone free, 250 mg/l cefotaxime, 5 mg/l PPT or bialaphos, pH 5.8

A. tumefaciens strain EHA 105 carrying binary vector was cultured on minimum AB medium (Chilton et al. 1974) supplemented with the following antibiotics: 100 μ g/ml spectinomycin, 300 μ g/ml streptomycin, and 60 μ g/ml rifampicin. Plates were cultured at 28 °C until individual colonies were visible. One colony was used to prepare a yeast extract-peptone (YEP) plate which was subsequently used to prepare YEP liquid culture 24 h before the transformation experiments. Next morning, *Agrobacterium* cultures were centrifuged (10 min 4000 rpm at room temperature), and pellets were resuspended with liquid filter-sterilized Chu infection medium supplemented with 15 g/l maltose and glucose, 1 g/l casamino acid, and 100 μ M

acetosyringone (pH 5.2). *VirG* genes were induced at 21 °C between 1.5 and 3 h. Individual embryogenic rice calli were inoculated with approximately 20 μ l *Agrobacterium* suspension, O.D. 0.5 (at room temperature) and after 45–50 min access, *Agrobacterium* suspension was blotted dry with sterile filter paper.

Cocultivation was carried out in darkness at 21 °C for 3 days. The infected embryogenic calli after washing with liquid Chu medium contain cefotaxime were transferred directly to Chu selection media supplemented with 3 mg/l PPT or bialaphos following by transfer to the fresh medium with 5 mg/l PPT or bialaphos after 15 days. All callus cultures were maintained in darkness at 24–26 °C.

Fig. 1 Schematic representation of the binary vector with *AtNCED3* gene

Well-growing calli in the presence of the selection agent were transferred to MS shoots induction medium containing 3 mg/l PPT or bialaphos supplemented with 1 mg/l naphthalene acetic acid (NAA) and 4 mg/l kinetin. Cultures were gradually moved from darkness into full light conditions with 12/12 h photoperiod and light intensity $1450 \mu\text{mol m}^{-2} \text{s}^{-1}$. Green shoots were rooted in the presence of 5 mg/l PPT or bialaphos on MS growth hormone free medium.

Molecular analyses

DNA extraction

Following growth under hydroponic conditions for 1 week, young leaves were collected for DNA extraction. The extraction was done according to the rice DNA extraction protocol by Dr. Mathias Lorieux, International Center for Tropical Agriculture (CIAT) (personal communication). Accordingly, 450 μl of extraction buffer (100 mM Tris-HCl, pH 8.0; 1.4 mM NaCl; 20 mM EDTA, pH 8.0; 1 % polyethylene glycol, wt 8000; 2 % alkyltrimethylammonium bromide, ≥ 95 %; 0.5 % sodium hydrogensulfite) were added to each tube containing 150 mg of ground leaf tissue and incubated at 74 °C for 30 min. Then, 480 μl of chloroform/isoamyl alcohol (24:1) was added and mixed for 5 min. After centrifugation at room temperature for 30 min (4000 rpm), the supernatant was transferred to a new tube and precipitated by using 270 μl isopropanol for 1 h at 20 °C. Samples were centrifuged at 10 °C for 30 min (4000 rpm), and the pellet was washed with 70 % ethanol and resuspended in nuclease free water. Re-precipitation with ammonium acetate provided good quality DNA suitable for Southern blot analysis.

Polymerase chain reaction analyses

Amplification of the bar gene region by conventional polymerase chain reaction (PCR) was done as an initial screening to select potential transgenic events with the following primers:

Bar-Fw-3 : 5'- GCACGCAACGCCTACGACTGG-3'
Bar-Rv-3 : 5'- TCAGATCTCGGTGACGGGACAG-3'

PCR conditions were performed with settings of initial denaturation at 95 °C for 2 min, 35 cycles each at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s with final extension at 72 °C for 5 min. PCR products were checked in 1 % agarose gel.

Southern blot analyses

In order to establish copy number of bar gene and to identify independent transgenic events, 10 μg DNA of each PCR-positive plant was digested using either EcoRI or SmaI (5U/ μg DNA). Because these enzymes only cut once into T-DNA without cutting the bar gene, they can be used to determine the copy number of bar gene. Digested DNA was separated in a 0.8 % agarose gel and blotted on a positively charged membrane nylon (Hybond-N+, Nylon Membranes, Positively Charged, *GE Healthcare Bio-Sciences Corp*). Membranes were hybridized with digoxigenin-labeled PCR probe for bar overnight and then incubated with the alkaline phosphate conjugated anti-digoxigenin antibody. After washing, blocking, and chemiluminescent reaction, the membranes were exposed to X-ray film for a minimum of 4 h.

Synthesis and labeling of probe was done following the instructions specified by the kit (PCR DIG Probe Synthesis Kit instructions, Roche). Primer bar used in PCR was also used to synthesize the bar DIG probe.

Integration of backbone sequences

PCR amplification of backbone regions linked to T-DNA was performed. For each border, three backbone regions were amplified ranging from the nearest region to the border to almost 1000 pb from border (Table 2). PCR conditions were performed with settings of initial denaturation at 95 °C for 2 min, 35 cycles at 94 °C for 30 s, annealing temperature (specific to each set of primers) for 30 s, and 72 °C for 30 sec or 1 min. Final extension was at 72 °C for 5 min. PCR products were checked in 1.2 % agarose gel.

Chlorophenol red test

A chlorophenol red assay was used to verify the bar gene activity in selected putative transgenic plants (Wright et al. 2001) and untransformed controls. Leaf segments were placed into individual wells of a multi-well plate containing half concentration of MS medium supplemented with 25 mg/l chlorophenol red, 3 mg/l bialaphos, and 8 g/l bacto agar, pH 6.2 and were incubated at 26 °C for 24 h.

PPT leaf painting test

The T0 plants and untransformed controls grown in the screen house were tested with 2 % aqueous solution of PPT containing 0.1 % (v/v) Tween-20 (Rasco-Gaunt et al. 1999) as well with aqueous solution of 0.1 % Tween-20. Leaf tissue response to PPT presence was scored after 1 week.

Table 2 Amplification of backbone regions

Primer	Sequence (5'-3')	Distance (bp) outside RB
RB 17 pb Rv	ACGCTCTTTTCTCTTAG	17
T-DNA 141 bp Fw	TAGCGCGCAAAGCTAGGATAAA	
RB 101 pb Rv	GAACCCTGTGGTTGGCAT	101
T-DNA 141 bp Fw	TAGCGCGCAAAGCTAGGATAAA	
RB 867 bp Rv	GATTAGCAGAGCGAGGTATGTAG	867
T-DNA 112 bp Fw	CGCGGTGTCATCTATGTTACTA	
Primer	Sequence (5'-3')	Distance (bp) outside LB
LB 20 pb Fw	GGGTGCAAAGCGGCAGCGGC	20
T-DNA 120 bp Rv	ATAACGCTGCGGACATCTAC	
LB 97 pb Fw	ACATGGCTCAGTTCTCAATGG	97
T-DNA 50 bp Rv	TGACCATCATACTCATTGCTGAT	
T-DNA 120 bp Rv	ATAACGCTGCGGACATCTAC	935
LB 935 bp Fw	GATCGACATTGATCTGGCTATCT	

Results

Effect of PPT and bialaphos on rice tissues

Prior to the transformation experiments, we tested untransformed Curinga tissue sensitivity to both selective agents, PPT and bialaphos, in Chu medium for callus proliferation and MS medium for shoots induction and rooting (Fig. 2). Parallel control cultures were carried out on media without selective agents (Fig. 2a–c). Three concentrations of PPT and bialaphos (1, 3, and 5 mg/l) were tested on callus proliferation in the Chu medium. The callus cultures were transferred to the fresh medium with increased concentration of PPT or bialaphos every 15 days. A strong tissue necrosis became visible after the second transfer (Fig. 2d). The shoots induction was originally tested with 10 and 20 mg/l PPT or bialaphos on MS medium. These concentrations were too high for Curinga calli so no shoots formed (Fig. 2e). Consequently, the LD 100 was determined for PPT and bialaphos as 3 mg/l for shoots induction and 5 mg/l for roots formation (Fig. 2f).

Transgenic plant selection

A minimum of four transformation experiments were performed with ten constructs where embryogenic calli from approximately 50 mature embryos were induced and subcultured. We selected 302 putative transgenic plants with either mixed selection scheme where both PPT and bialaphos were used or where only one selection agent was used through the entire selection process. Callus selection was done on Chu medium supplemented with 3 mg/l PPT or bialaphos. Fifteen days following selection, pressure was increased to 5 mg/l in the second subculture. We also performed experiments with two calli subcultures with 3 mg/l PPT, followed by a third one with 5 mg/l PPT in 15-day intervals.

When we statistically analyzed (chi-square test using SAS version 9.2 on the Linux platform) the number of transgenic plants obtained from these two different selection schemes, the results showed no statistically significant difference between the experiments. Furthermore, there was no statistically significant difference between the experiments carried out with PPT, bialaphos, or with the mix of both selection agents during the entire selection process (data not shown).

When we analyzed the number of calli needed to produce one transgenic plant depending on the construct and selection agent, the results showed significant differences (Table 3). The PPT selection worked better with the following constructs p7i2xU-*AVP1*, *AtNCED3*, p7i2xU-*DA1*, and p7i2xU-*TOR* in difference bialaphos was better for *AtGLU2*, p7i2xU-*Lsi1*, p7i2xU-*PLD-alpha*, and both p7i2xU-*Rubisco* genes. The mixed selection worked similar to the PPT only for p7i2xU-*AVP1* construct. For the shoots induction, we used MS medium supplemented with 3 mg/l PPT or bialaphos and for rooting the concentration of selection agents was increased to 5 mg/l (Fig. 3a–d).

Molecular analyses of transgenic plants

Overall, we analyzed 273 T0 plants using Southern blot analysis of which 134 transgenic events from ten constructs were confirmed while 73 plants carried only a single copy of the transgene (Table 4). The transformation efficiency (TE) for all constructs used represented 49 % when TE was calculated as the number of T0 plants selected in vitro and transgenic plants identified by Southern blot analyses. Overall, 45 events from nine constructs were selected on PPT alone during the entire selection process and 54 events from ten constructs were obtained on bialaphos alone. In contrast, only 24 events were selected with the mixed selection schema.

Fig. 2 Curinga tissues' sensitivity test. **a** Callus induction on Chu medium, control. **b** Shoots induction on MS medium, control. **c** Callus induction with 3 mg/l bialaphos. **d** Shoots induction with 3 mg/l bialaphos. **e** Rooting on MS medium, control. **f** Rooting with 5 mg/l bialaphos (scale bar in mm)

PCR of bar gene

From 321 Curinga rice plants regenerated and analyzed by PCR, 143 showed amplification of the bar gene (Table 4 and Fig. 4a) and were considered as transgenic or PCR positive. Amplification of a band of 318 bp confirmed the transgenic status of plants. This first screening through PCR allowed plant selection for subsequent molecular characterization through Southern blot.

Southern blot analyses

Through Southern blot analysis, 134 different transgenic events have been identified. Copies of the bar gene integrated in rice genome ranged from one to several (Table 4 and

Fig. 5). The maximum copy number of transgene integrated in rice plants were five. Of the total events, 77 had one copy of bar gene which represents 57.46 %, and 29 events had two copies which represents 21.64 %. Our results showed that in total 79.1 % of all transgenic events obtained harbored low copy number of the transgene. Only 16.41 % of events harbored three copies, and 4.47 % events were considered as multi-copy events (≥ 4 bar gene copies).

Backbone regions linked to T-DNA

Five pairs of primers covering regions outside the T-DNA borders were used in different PCRs to determine if there was integration of backbone sequences. One hundred and one transgenic plants belonging to all constructs and

Table 3 Number of calli needed to obtain one transgenic plants when PPT (PPT), bialaphos (BIA), or mixture of both selection agents (PPT-BIA) have been used

Selection agent	Construct	F^a	Number of calli
BIA	p7i2xU- <i>AVP1</i>	0.01603	186
PPT	p7i2xU- <i>AVP1</i>	0.03475	85
PPT-BIA	p7i2xU- <i>AVP1</i>	0.03333	89
BIA	p7i2xU- <i>AtGLU2</i>	0.00493	607
PPT	p7i2xU- <i>AtGLU2</i>	0.00402	745
BIA	p7i2xU- <i>AtNCED3</i>	0.01542	193
PPT	p7i2xU- <i>AtNCED3</i>	0.02370	125
PPT-BIA	p7i2xU- <i>AtNCED3</i>	0.00164	1829
BIA	p7i2xU- <i>DA1</i>	0.00503	595
PPT	p7i2xU- <i>DA1</i>	0.03659	81
PPT-BIA	p7i2xU- <i>DA1</i>	0.01316	227
BIA	p7i2xU- <i>Lsi1</i>	0.02841	104
PPT	p7i2xU- <i>Lsi1</i>	0.01786	167
PPT-BIA	p7i2xU- <i>Lsi1</i>	0.01427	209
BIA	p7i2xU- <i>PLD-alpha</i>	0.05556	53
PPT	p7i2xU- <i>PLD-alpha</i>	0.01282	233
PPT-BIA	p7i2xU- <i>PLD-alpha</i>	0.00714	418
BIA	p7i2xU- <i>Rubisco1</i>	0.01786	167
PPT	p7i2xU- <i>Rubisco1</i>	0.02139	139
PPT-BIA	p7i2xU- <i>Rubisco1</i>	0.01205	248
BIA	p7i2xU- <i>Rubisco2</i>	0.04545	65
PPT	p7i2xU- <i>Rubisco2</i>	0.00778	384
PPT	p7i2xU-TOR	0.04712	63
PPT-BIA	p7i2xU-TOR	0.01613	185

^a Treatment means were compared using 95 % confidence intervals

representing unique events were analyzed by PCR (Table 5 and Fig. 4b). For each border, three backbone regions were amplified demonstrating varying distances from each border. As shown in Table 5, in the case of the right border (RB), 25.7 % (26) of the total transgenic plants analyzed showed PCR amplification of backbone sequences linked to the RB. In the case of the left border (LB), 35.6 % (36) plants showed amplification of backbone regions linked to the LB. In summary, 40.6 % (41) plants had backbone insertions linked to T-DNA (either to RB or LB). Of those 41, less than half had backbone integration from both borders (48.7 %). From all lines that were analyzed, a total of 25 lines (24.75 %) contained insertion toward right border and 36 lines (35.64 %) contained backbone insertion toward left border (data not shown).

Chlorophenol red test

The color of the medium in this assay changed from purple to yellow indicating that the bar gene activity in transformed rice

cells acidified the medium by metabolizing bialaphos. We tested leaf pieces from 45 putative transgenic shoots and untransformed control plants for bar gene expression using the chlorophenol red test (CRT; data not shown). In six cases, the putative transgenic shoots were false negative as determined by the Southern blot analysis, which indicated they were transgenic, carrying the single copy of the transgene. Another plant was identified as negative by CRT; however, it was later identified by Southern blot as transgenic with three copies of the transgene. In contrary, two plants scored positive by CRT but were negative by Southern blot. The results obtained from the CRT and Southern blot showed a 13 % mismatch.

Leaf brush test with PPT

To evaluate the herbicide resistance level, part of the leaves of transgenic plants and untransformed controls were painted with an aqueous solution of PPT supplemented with Tween-Os20. Overall, 23 transgenic plants from eight constructs (p7i2x-*AVP1*, p7i2x-*Lsi1*, p7i2x-*DA1*, p7i2x-*Rubisco1*, p7i2x-*Rubisco2*, p7i2x-*TOR*, p7i2x-*AtNCED3*, and p7i2x-*GLU2*) showed complete tolerance, i.e., treated leaves stayed green without visible damage, regardless of the transgene copy numbers integrated in the plant genome. However, the leaves of untransformed plants were completely necrotic (data not shown).

T0 plants and T1 seeds

All transgenic events (107 plants) with low copy numbers integrated in rice genome determined by Southern blot analyses were transferred to the screen house (data not shown). Flowering panicles were covered with the pollination bag to prevent cross pollination, and T0 plants were harvested, additionally dried, and manually trashed. T1 seeds from individual plant were cleaned and stored at 10 °C for the following phenotypic experiments.

Discussion

The bar gene isolated from *Streptomyces hygroscopicus* encoding the phosphinotricine acetyltransferase (PAT) enzyme which allows resistance to herbicides containing PPT (Thompson et al. 1987, Schomburg and Schomburg 2009) can be used for selection of transgenic plants as initially demonstrated by De Block et al. (1989) for tobacco, potato, and tomato plants. Bialaphos (Ogawa et al. 1973) is a tripeptide antibiotic consisting of PPT, an analog of L-glutamic acid and two L-alanine residues and is produced by fermentation of *Streptomyces hygroscopicus* (Bayer et al. 1972) by

Fig. 3 Curinga transgenic tissues. **a** p7i2xU-*Lew2* gene callus proliferation with 3 mg/l bialaphos. **b** p7i2xU-*Rubisco 1* gene shoots induction with 3 mg/l bialaphos. **c** p7i2xU-*GLU2* gene shoots elongation with 3 mg/l bialaphos. **d** p7i2xU-*AtNCED3* gene rooting with 5 mg/l bialaphos (scale bar in mm)

Herbiace®, Meiji Seika Ltd. When these residues are removed by peptidases, PPT is able to inhibit glutamine synthetase (GS), the enzyme central to assimilation of ammonium and regulation of nitrogen metabolism (Mifflin and Lea 1977). If the GS is inhibited by PPT, accumulation of ammonia results in death of the plant cell (Tachibana et al. 1986). PPT is chemically synthesized by Basta®, Hoechst AG, Germany 1975 (currently Sanofi-Aventis, 2004).

The first herbicide-resistant transgenic rice plants with the bar gene were produced by electroporation (Dekeyser et al.

1989; Toki et al. 1992) by polyethylene glycol (PEG)-mediated transformation of rice protoplast (Datta et al. 1990) or by cell suspension cultures transformed via particle bombardment (Cao et al. 1992; Xu et al. 1996).

Agrobacterium-mediated transformation using the bar gene in combination with anti-necrotic treatments was reported for *japonica* rice R-321 by Enriquez-Obregon et al. (1999). The following authors used GFP and bar selection with different *japonica* rice genotypes (Jang et al. 1999; Nakamura et al. 2010) or in combination with transformation histone enhancer

Table 4 Molecular analyses of ten constructs using Southern blot analysis

Construct	No. of putative transgenic plants	No. of plants analyzed by Southern blot analysis	No. of PCR-positive plants	No. of transgenic events	1 copy	2 copies	3 copies	4 copies	Multi-copy
p7i2x- <i>AtNCED3</i>	46	33	25	20	9	6	3	2	0
p7i2x- <i>Lsi1</i>	63	58	34	29	14	9	6	0	0
p7i2x- <i>DA1</i>	20	18	5	11	8	2	1	0	0
p7i2x- <i>AVP1</i>	49	38	16	19	11	4	2	1	1
p7i2x- <i>Rubisco1</i>	27	26	12	10	5	1	2	2	0
p7i2x- <i>AtGLU2</i>	7	5	3	2	1	0	1	0	0
p7i2x- <i>LEW2</i>	35	32	13	15	8	4	3	0	0
p7i2x- <i>PLD-alpha</i>	22	18	6	9	6	2	1	0	0
p7i2x- <i>Rubisco2</i>	25	20	7	7	3	1	3	0	0
p7i2x- <i>TOR</i>	27	25	22	12	12	0	0	0	0
Total	321	273	143	134	77	29	22	5	1

Fig. 4 Molecular analysis through conventional PCR. **a** PCR amplification of bar gene. Lanes 2–9, 11–18: PCR-positive plants. Lane 20: plasmid transgenic control. Lanes 21 and 22: genomic transgenic controls. Lane 23: not transgenic control. Lane 24: reaction control. Lanes 1, 10, and 19: 1-Kb Plus ladder (Invitrogen). Size of band, 318 bp. **b** PCR amplification of backbone integration. Lanes 1 and 24: 1-Kb Plus ladder (Invitrogen). Lanes 2–23: transgenic plants under backbone analysis.

Lane 25: plasmid p7i2x-*AtNCED3*. Lane 26: plasmid p7i2x-*PLD-alpha*. Lanes 27–29: not transgenic controls. Lane 30: reaction control. Size of bands: 279 bp in constructs p7i2x-*PLD-alpha* and p7i2x-*Rubisco1* and 242 bp in remaining plasmids. Note the size difference between plants transformed either with constructs p7i2x-*PLD-alpha* and p7i2x-*Rubisco2* (Lanes 2, 7, 8, and 11) or remaining plasmids (Lanes 4, 5, 12–15)

(Zheng et al. 2009). Recently, Duan et al. (2012) reported a high-throughput protocol for *japonica* cv. Nipponbare and Wanjiang 97 with mannose and bar selection. It is difficult to compare our results with those previously published due to the different rice genotypes used as well as promoters driving the bar gene. Furthermore, we observed big differences among our experiments when we compared the number of calli

needed for one transgenic plant production if comparing the ten constructs with which we worked.

In our study, the majority (73.1 %) of the transgenic events produced using *Agrobacterium*-mediated transformation at most two copies of the transgene. This confirms that transformation mediated by *Agrobacterium* favors the regeneration of low copy transgenic plants. Previous studies support this finding, as demonstrated in maize, where Shou et al. (2004) obtained regeneration of 92 % of low copy number events (less than three copies) and sorghum where Zhou et al. (2000) reported 72 % of single-copy events by using *Agrobacterium*. Dai et al. (2001) also reported the regeneration of low copy events in rice transgenic plants. These reports confirm the central role of *Agrobacterium* in efficient transformation protocols for agronomically important cereal crops such as rice, wheat, maize, barley, and sorghum (Cheng et al. 2006).

Fig. 5 Southern blot detecting bar gene copy number in transgenic rice plants. Lane 1: not transgenic control. Lanes 2–5: single-copy events. Lane 6: two-copy event. Lane 7: genomic DNA. Lanes 2 and 4, the same transformation event

Table 5 PCR analyses of 101 transgenic plants

No. of transgenic events with only backbone integrated outside RB	No. of transgenic events with only backbone integrated outside LB	No. of transgenic events with integrated backbone outside RB and LB	No. of transgenic events with no backbone sequence integrated
6	15	20	60

A large portion (40.6 %) of rice transgenic plants obtained in our study had backbone sequences linked to T-DNA. Previous studies identified vector backbone sequences linked to T-DNA in a range of species transformed by *Agrobacterium* including barley (Lange et al. 2006), Arabidopsis (De Buck et al. 2000), tobacco (Kononov et al. 1997 and De Buck et al. 2000), wheat (Wu et al. 2006) and rice (Kim et al. 2003; Afolabi et al. 2004; Kuraya et al. 2004 and Yin and Wang 2000). These reports also show significant ratios of backbone insertions ranking from 20 % (De Buck et al. 2000) to 75 % (Kononov et al. 1997) in transgenic plants not including rice and from 33 % (Yin and Wang 2000) to 92 % (Kuraya et al. 2004) in rice transgenic plants. One explanation for the integration of vector backbone appears to be inefficient recognition of the left and right border as initiation and termination sites for T-DNA transfer resulting in read-through at both borders (De Buck et al. 2000). Fang et al. (2000), in study done on rice, identified two types of vector backbone sequence: directly linked to the T-DNA across either LB or RB (type I) and backbone sequence not directly linked to a T-DNA border (type II). In our study, we focused only on type I backbone sequences integration. We found 40.6 % of this type of insertion, and it is only slightly higher compared to what has previously been reported (37.5 %). Moreover, as it was showed by Zuniga, 2014, CIAT (personal communication), using the chromosome walking approach, the adjacent region of the T-DNA integration in rice can have different configuration closed to RB.

The original chlorophenol red assay described by Kramer et al. (1993) for maize callus selected with PPT was modified for transgenic plants selected with D-mannose for maize and wheat (Wright et al. 2001), for *japonica* rice (Lucca et al. 2001), for chickpea (Patil et al. 2009), and for transgenic cowpea plants (Bakshi et al. 2012). All of these authors support the high correlation between the CRT and PCR results. Based on our Southern blot results compared to the CRT, we found discrepancies in 13 % of the T0 plants tested. The bar gene expression in T0 rice plants together with the herbicide resistance of transgenic plants can be tested by the simple leaf painting test, using PPT, bialaphos, or the herbicides Basta (Rathore et al. 1993) and Challenge (Wu et al. 2003). As we found, the results from this assay were highly correlated with the PCR results (Rasco-Gaunt et al. 1999) where all transgenic plants tested positive by PCR with copy number identified by Southern blot analyses. Positive correlation between molecular analyses and bar gene expression resulting herbicide resistance was also described in other crops, for example wheat (Wu et al. 2006), carrot (Jayaraj et al. 2008), or cassava (Koehorst-van Putten et al. 2012).

By producing over hundreds of transgenic events carrying different genes of interest, we created a good basis for the further phenotyping experiments to evaluate the performance for agronomically important traits, as well as we have

developed a transformation protocol for an important commercial Brazilian upland rice cultivar with herbicide resistance.

Acknowledgments We would like to acknowledge funding for this work from the EMBRAPA-Monsanto grant, Brasilia. We are also grateful to Mr. G. Delgado for his excellent technical assistance with the rice tissue cultures. Mr. D. Marin for his help with the T0 plants growth and the T1 seeds handling and Mrs. Miriam Duque for the statistical analyses.

Conflict of interest The authors declare that they have no conflict of interest.

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

References

Blackwell Publishing, Ltd.

- Afolabi A, Worland B, Snape W, Vain P (2004) A large-scale study of rice plants transformed with different T-DNAs provides new insights into locus composition and T-DNA linkage configurations. *Theor Appl Genet* 109:815–826
- Bajaj S, Mohanty A (2005) Recent advances in rice biotechnology—towards genetically superior transgenic rice. *Plant Biotechnol J* 3: 275–307
- Bakshi S, Saha B, Roy NK, Mishra S, Panda K, Sahoo L (2012) Successful recovery of transgenic cowpea (*Vigna unguiculata*) using the 6-phosphomannose isomerase gene as the selectable marker. *Plant Cell Rep* 31:1093–1103
- Bayer E, Gugel KH, Hägele K, Hagenmaier H, Jessipow S, König WA, Zähler H (1972) Metabolic products of microorganisms. Phosphinothricin and phosphinothricyl-alanyl-analine. *Helv Chim Acta* 55:224–239
- Campbell CA, Myers RJK, Curtin D (1995) Managing nitrogen for sustainable crop production. *Fertil Res* 42:277–296
- Cao J, Duan XL, McElroy D, Wu R (1992) Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. *Plant Cell Rep* 11:586–591
- Chen H, Lin Y, Zhang Q (2010a) Rice. In: Kempker F, Jung C (ed) Genetic modification of plants. *Biotechnol Agricult For* 64:423–451
- Chen Z, Hong X, Zhang H, Wang Y, Li X, Zhu J-K, Gong Z (2005) Disruption of the cellulose synthase gene, AtCesA8/IRX1, enhances drought and osmotic stress tolerance in Arabidopsis. *Plant J* 43:273–283
- Chen Y-T, Fang Q-S, Chiang C-H, Yeh S-D, Wu H-W, Yu T-A (2010b) Transgenic *Eustoma grandiflorum* expressing the bar gene are resistant to the herbicide Basta®. *Plant Cell Rep* 102:347–356
- Cheng M, Lowe B, Spencer M, Ye X, Armstrong C (2006) Invited review: factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. *In Vitro Cell Dev Biol Plant* 40:31–45
- Chu C-C (1978) The N6 medium and its application to another culture of cereal crops. *Proc Symp Plant Tissue Culture*, Science Press, Peking, pp. 43–50

- Charity JA, Holland L, Grace J, Walter C (2005) Consistent and stable expression of the *nptII*, *uidA* and *bar* genes in transgenic *Pinus radiata* after *Agrobacterium tumefaciens*-mediated transformation using nurse cultures. *Plant Cell Rep* 23: 606–616
- Chilton M-D, Currier T, Farrand S, Bendish A, Gordon M, Nester E (1974) *Agrobacterium tumefaciens* DNA and PS bacteriophage DNA not detected in crown gall tumors. *Proc Natl Acad Sci U S A* 71:3672–3676
- Coschigano KT, Melo-Oliveira R, Lim J, Coruzzi GM (1998) New Arabidopsis *gls* mutants and distinct Fd-GOGAT Genes: implications for photorespiration and primary nitrogen assimilation. *Plant Cell* 10:741–752
- Dai S, Zheng P, Marmey P, Zhang S, Tian W, Chen S, Beachy RN, Fauquet C (2001) Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment. *Mol Breed* 7:25–33
- Deikman J, Petracek M, Heard JE (2011) Drought tolerance through biotechnology: improving translation from the laboratory to farmers' fields. *Curr Opin Biotechnol* 23:1–8
- Datta SK, Peterhans A, Datta K, Potrykus I (1990) Genetically engineered fertile indica-rice recovered from protoplasts. *Bio/Technology* 8:736–740
- De Block M, De Brouwer D, Tenning T (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. *Plant Physiol* 9:694–701
- De Buck S, De Wilde C, Van Montagu M, Depicker A (2000) T-DNA vector backbone sequences are frequently integrated into the genome of transgenic plants obtained by *Agrobacterium*-mediated transformation. *Mol Breed* 6:459–468
- Dekeyser R, Claes B, Marichal M, Van Montagu M, Caplan A (1989) Evaluation of selectable markers for rice transformation. *Plant Physiol* 90:217–223
- Deprost D, Yao L, Sormani R, Moreau M, Leterreux G, Nicolai M, Bedu M, Robaglia C, Meyer C (2007) The Arabidopsis TOR kinase links plant growth, yield, stress resistance and mRNA translation. *EMBO Rep* 8:864–870
- Duan Y, Zhai Ch H, Li J, Mei W, Gui H, Ni D, Song F, Li L, Zhang W, Yang J (2012) An efficient and high-throughput protocol for *Agrobacterium*-mediated transformation based on phosphomannose isomerase positive selection in Japonica rice (*Oryza sativa* L.). *Plant Cell Rep* 31:1611–1624
- Enriquez-Obregon GA, Prieto-Samsonov DL, de la Riva GA, Perez M, Selman-Housein G, Vazquez-Padron RI (1999) *Agrobacterium*-mediated Japonica rice transformation: a procedure assisted by an antinecrotic treatment. *Plant Cell Tissue Org Cult* 59:159–168
- Evans R (1989) Photosynthesis and nitrogen relationships in leaves of C3 plants. *Oecologia* 78:9–19
- Fageria NK (2002) Nutrient management for improving upland rice productivity and sustainability. *Communication in Soil Science and Plant Analyses* 32:26603–2629
- Fageria NK (2009) The use of nutrients in crop plants. CRC Press, New York
- Fageria NK (2010) Optimal nitrogen fertilization for upland rice. World Congress of Soil Solutions for a Changing World. 1–6 August, 2010, Brisbane, Australia
- Fang J, Zhai WX, Wang WM, Zhu LH (2000) Amplification and analysis of T-DNA flanking sequences in transgenic rice lines. *Rice Genet Newsl* 17:112–114
- FAO (2013) www.fao.org
- FAOSTAT (2012) The Statistics Division of the FAO, available at: <http://faostat.fao.org>
- Gaudin ACM, Henry A, Sparks AH, Slamet-Loedin IH (2013) Taking transgenic rice drought screening to the field. *J Exp Bot* 64:109–118
- Gaxiola RA, Li J, Undurraga S, Dang LM, Allen GJ, Alper SL, Fink GR (2001) Drought- and salt-tolerant plants result from overexpression of the AVP1 H1-pump. *Proc Natl Acad Sci U S A* 98:11444–11449
- Hadiarto T, Tran L-S P (2011) Progress studies of drought-responsive genes in rice. *Plant Cell Rep* 30:297–310
- Hiei Y, Ohta S, Komari T, Kumashiro T (1994a) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* 6:271–282
- Hu F, Zhang L, Wang X, Ding J, Dianxing, Wu D (2005) *Agrobacterium*-mediated transformed transgenic triploid bermudagrass (*Cynodon dactylon* × *C. transvaalensis*) plants are highly resistant to the glufosinate herbicide Liberty. *Plant Cell Tissue Org Cult* 83:13–19
- Imai K, Suzuki Y, Mae T, Makino A (2008) Changes in the synthesis of Rubisco in rice leaves in relation to senescence and N influx. *Ann Bot* 101:135–144
- Iuchi S, Kobayashi M, Ti T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. *Plant J* 27:325–333
- Jang I-C, Nahm BH, Kim J-K (1999) Subcellular targeting of green fluorescent protein to plastids in transgenic rice plants provides a high-level expression system. *Mol Breed* 5:453–466
- Jayaraj J, Devlin JR, Punj Z (2008) Metabolic engineering of novel ketocarotenoid production in carrot plants. *Transgenic Res* 17: 489–501
- Jeong JS, Kim YS, Redillas MCFR, Jang G, Jung H, Bang SW, Choi YD, Ha SH, Rousseau C, Kim JK (2013) OsNAC5 overexpression enlarges root diameter in rice plants leading to enhanced drought tolerance and increased grain yield in the field. *Plant Biotechnol J* 11:101–114
- Joyce P, Kuwahata M, Turner N, Lakshmanan P (2010) Selection system and co-cultivation medium are important determinants of *Agrobacterium*-mediated transformation of sugarcane. *Plant Cell Rep* 29:173–183
- Kamo K, Young HY (2009) Long-term gus expression from *Gladiolus* callus lines containing either a *bar-uidA* fusion gene or *bar* and *uidA* delivered on separate plasmids. *Plant Cell Tiss Organ Cult* 98:263–272
- Kim S, Lee J, Jun S, Park S, Kang H, Kwon S, An G (2003) Transgene structures in T-DNA-inserted rice plants. *Plant Mol Biol* 52:761–773
- Knapp EJ, Kauch AP, Chandless JM (2000) Transformation of three genera of orchid using the *bar* gene as a selection marker. *Plant Cell Rep* 19:893–898
- Khuong TTH, Crete P, Robaglia C, Caffari S (2013) Optimisation of tomato Micro-tom regeneration and selection on glufosinate/Basta and dependency of gene silencing on transgene copy number. *Plant Cell Rep* 32:1441–145
- Kopertekh L, Broer I, Schiemann J (2009) Developmentally regulated site-specific marker gene excision in transgenic *B. napus* plants. *Plant Cell Rep* 28:1075–1083
- Koehorst-van Putten HJJ, Sudarmonowati E, Herman M, Pereira-Bertram IJ, Wolters AMA, Meima H, de Vetten N, Raemakers CJJM, Visser RGF (2012) Field testing and exploitation of genetically modified cassava with low-amylose or amylose-free starch in Indonesia. *Transgenic Res* 21:39–5
- Kononov M, Bassuner B, Gelvin S (1997) Integration of T-DNA binary vector backbone sequences into tobacco genome: evidence for multiplex complex patterns of integration. *Plant J* 11:945–957
- Kramer C, DiMaio J, Carswell GK, Shilito R (1993) Selection of transformed protoplast-derived *Zea mays* colonies with phosphinothricin and a novel assay using the pH indicator. *Planta* 190:454–458

- Kuai B, Perret S, Wan SM, Dalton SJ, Bettany AJE, Morris P (2001) Transformation of oat and inheritance of bar gene expression. *Plant Cell Tissue Org Cult* 66:79–88
- Kuraya Y, Ohta S, Fukuda M, Hiei Y, Murai N, Hamada K, Ueki J, Imaseki H, Komari T (2004) Suppression of transfer of non-T-DNA ‘vector backbone’ sequences by multiple left border repeats in vectors for transformation of higher plants mediated by *Agrobacterium tumefaciens*. *Mol Breed* 14:309–320
- Lancien M, Martin M, Hsieh MH et al (2002) *Arabidopsis glt1*-T mutant defines a role for NADH-GOGAT in the non-photorespiratory ammonium assimilatory pathway. *Plant J* 29:347–358
- Lange M, Vincze E, Möller M, Holm P (2006) Molecular analysis of transgene and vector backbone integration into the barley genome following *Agrobacterium*-mediated transformation. *Plant Cell Rep* 25:815–820
- Lawlor DW (2013) Genetic engineering to improve plant performance under drought: physiological evaluation of achievements, limitations, and possibilities. *J Exp Bot* 64:83–108
- Li Y, Zheng L, Corke F, Smith C, Bevan MW (2008) Control of final seed and organ size by the *DAL* gene family in *Arabidopsis thaliana*. *Genes Dev* 22:1331–1336
- Lucca P, Ye X, Potrykus I (2001) Effective selection and regeneration of transgenic rice plants with mannose as selective agent. *Mol Breed* 7: 43–49
- Ma JM, Tamai K, Yamaji N, Mitani N, Konishi S, Katsuhara M, Ishiguro M, Murata Y, Yano M (2006) A silicon transporter in rice. *Nature* 440:688–691
- McCabe MS, Schepers F, van der Arend A, Mohapatra U, de Laat AMM, Power JB, Davey MR (1999) Increased stable inheritance of herbicide resistance in transgenic lettuce carrying a *petE* promoter-bar gene compared with a CaMV 35S-bar gene. *Theor Appl Genet* 99:587–592
- Menand B, Desnos T, Nussaume L, Berger F, Bouchez D, Meyer C, Robaglia C (2002) Expression and disruption of the *Arabidopsis* TOR (target of rapamycin) gene. *Proc Natl Acad Sci U S A* 99: 6422–6427
- Mifflin BJ, Lea PJ (1977) Amino acid metabolism. *Annu Rev Plant Physiol* 28:299–329
- Mishra G, Zhang W, Deng F, Zhao J, Wang X (2006) A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in *Arabidopsis*. *Science* 322:264–266
- Miyazaki N, Ueno O, Saitou K (2013) Effects of nitrogen on the expression of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit multigene family members in Rice (*Oryza sativa* L.). *Plant Prod Sci* 16:37–40
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:47–497
- Nakamura S, Mano S, Tanaka Y, Ohnishi M, Nakamori C, Araki M, Niwa T, Nishimura M, Kaminaka H, Nakagawa T, Sato Y, Ishiguro S (2010) Gateway binary vectors with the Bialaphos resistance gene, *bar*, as a selection marker for plant transformation. *Biosci Biotechnol Biochem* 74:1315–1319
- Ogawa Y, Tsuruoka T, Inoue T, Niida S (1973) Studies on a new antibiotic SF-1293. II. Chemical structure of antibiotic SF-1293. *Sci Rep Meiji Seika Kaisha* 13:42–48
- Pasapula V, Shen G, Kuppu S, Paez-Valencia J, Mendoza M, Hou P, Chen J, Qiu X, Zhu L, Zhang X, Auld D, Blumwald E, Zhang H, Gaxiola R, Payton P (2011) Expression of an *Arabidopsis* vacuolar H⁺-pyrophosphatase gene (AVP1) in cotton improves drought- and salt tolerance and increases fiber yield in the field conditions. *Plant Biotechnol J* 9:88–99
- Patil G, Deokar A, Jain PK, Thengane RJ, Srinivasan R (2009) Development of a phosphomannose isomerase-based *Agrobacterium*-mediated transformation system for chickpea (*Cicer arietinum* L.). *Plant Cell Rep* 28: 1669–1676
- Patel M, Dewey RE, Qu R (2013) Enhancing *Agrobacterium tumefaciens*-mediated transformation efficiency of perennial ryegrass and rice using heat and high maltose treatments during bacterial infection. *Plant Cell Tissue Org Cult* 114:19–29
- Rasco-Gaunt S, Riley A, Lazzeri P, Barcelo P (1999) A facile method for screening for phosphinothricin (PPT) resistant transgenic wheats. *Mol Breed* 5:255–262
- Rathore KS, Chowdhury VK, Hodges TK (1993) Use of bar as a selectable marker gene and for the production of herbicide-resistant rice plants from protoplasts. *Plant Mol Biol* 21:871–884
- Schomburg D, Schomburg I (2009) Springer handbook of enzymes. Supplement Volume S2. Springer, Berlin, Heidelberg, New York
- Serraj J, Bennett J, Hardy B (2008) Drought frontiers in rice: crop improvement for increased rain fed production. IRRI, 400 p. World Scientific Publishing, Los Banos, Philippines
- Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. *J Exp Bot* 58:221–227
- Shou H, Frame B, Whitham S, Wang K (2004) Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*-mediated transformation. *Mol Breed* 13:201–208
- Sonia, Saini R, Singh RP, Jaiwal PK (2007) *Agrobacterium tumefaciens* mediated transfer of *Phaseolus vulgaris* α -amylase inhibitor-1 gene into mungbean *Vigna radiata* (L.) Wilczek using *bar* as selectable marker. *Plant Cell Rep* 26:187–198
- Suzuki Y, Ohkubo M, Hatakeyama H, Ohashi K, Yoshizawa R, Kojima S, Hayakawa T, Yamaya T, Mae T, Makino A (2007) Increased Rubisco content in transgenic rice transformed with the ‘Sense’ *rbcS* gene. *Plant Cell Physiol* 48:623–637
- Tachibana K, Watanaba T, Seikizawa Y, Takematsu T (1986) Accumulation of ammonia in plants treated with *bialaphos*. *J Pestic Sci* 11:33–37
- Taylor N (2008) Cellulose biosynthesis and deposition in higher plants. *New Phytol* 278:239–252
- Thompson CJ, Movva NR, Tizard R, Crameri R, Davies JE, Lauwereys M, Botterman J (1987) Characterization of the herbicide-resistance gene bar from *Streptomyces hygroscopicus*. *EMBO J* 6:2519–2523
- Toki S, Takamatsu S, Nojiri C, Ooba S, Anzai H, Iwata M, Christensen AH, Quail PH, Uchimiya H (1992) Expression of a maize ubiquitin gene promoter-bar chimeric gene in transgenic rice plants. *Plant Physiol* 100:1503–1507
- Twyman RM, Stoger E, Kohli A, Capell T, Christou P (2002) Selectable and screenable markers for rice transformation. In: Jackson JF, Linskens HF, Inman RB (eds) *Molecular methods of plant analysis. Testing for genetic manipulation in plants*, vol 22. Springer, Berlin, Heidelberg
- Valdez-Ortiz A, Medina-Godoy S, Valverde ME, Paredes-López O (2007) A transgenic tropical maize line generated by the direct transformation of the embryo-scutellum by *A. tumefaciens*. *Plant Cell Tissue Org Cult* 91:201–214
- Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Engineering drought tolerance in plants: discovering and tailoring genes unlock the future. *Curr Opin Biotechnol* 17:113–122
- Uraji M, Katagiri T, Okuma E, Ye W, Hossain MA, Masuda C, Miura A, Nakamura Y, Mori IC, Shinozaki K, Murata Y (2012) Cooperative function of *PLDa* and *PLDa1* in abscisic acid induced stomatal closure in *Arabidopsis*. *Plant Physiol* 199:450–460
- Weeks JT, Anderson OD, Blechl A (1993) Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum* L.). *Plant Physiol* 102:1077–1084
- Wen-Sun S, Forde BG (1989) Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. *Nucleic Acids Res* 17:8383
- Wright M, Dawson J, Dunder E, Suttie J, Reed J, Kramer C, Chang Y, Novitzky R, Wang H, Artim-Moore L (2001) Efficient biolistic transformation of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) using the phosphomannose isomerase gene, *pmi*, as the selectable marker. *Plant Cell Rep* 20:429–436
- Wu H, McCormac AC, Elliott MC, Chen D-F (1998) *Agrobacterium*-mediated stable transformation of cell suspension cultures of barley (*Hordeum vulgare*). *Plant Cell Tissue Org Cult* 54:161–171

- Wu H, Doherty A, Jones HD (2008) Efficient and rapid *Agrobacterium*-mediated genetic transformation of durum wheat (*Triticum turgidum* L. var. *durum*) using additional virulence genes. *Transgenic Res* 17: 425–436
- Wu H, Sparks CA, Jones HD (2006) Characterization of T-DNA loci and vector backbone sequences in transgenic wheat produced by *Agrobacterium*-mediated transformation. *Mol Breeding* 18:195–208
- Wu H, Sparks CA, Amoah B, Jones HD (2003) Factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat. *Plant Cell Rep* 21:659–668
- Xu D, Duan X, Wang B, Hong B, Ho T-HD, Wu R (1996) Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol* 110:249–257
- Yamaji N, Ma JF (2007) Spatial distribution and temporal variation of the rice silicon transporter *Lsi1*. *Plant Physiol* 143:1306–1313
- Yang S, Vanderbeld B, Wan J, Huang Y (2010) Narrowing down the targets: towards successful genetic engineering of drought-tolerance crops. *Mol Plant* 3:469–490
- Yin Z, Wang G (2000) Evidence of multiple complex patterns of T-DNA integration into the rice genome. *TAG* 100:461–470
- Zang N, Zhai H, Gao S, Chen W, He S, Liu Q (2009) Efficient production of transgenic plants using *bar* gene for herbicide resistance in sweetpotato. *Sci Horticul* 122:649–653
- Zeng P, Vadenais DA, Zhang Z, Polacco JC (2004) Refined glufosinate selection in *Agrobacterium* mediated transformation of soybean [*Glycine max* (L.) Merrill]. *Plant Cell Rep* 22:478–472
- Zhang S, Warkentin D, Sun B, Zhong H, Sticklen M (1996) Variation in the inheritance of expression among subclones for unselected (*uidA*) and selection (*bar*) transgenes in maize (*Zea mays* L.). *Theor Appl Gen* 92:752–761
- Zheng Y, He X-W, Ying Y-H, Lu J-F, Gelvin SB, Shou H-X (2009) Expression of the *Arabidopsis thaliana* Histone gene *AtHTA1* enhances rice transformation efficiency. *Mol Plant* 2:832–837
- Zhou Z, Cai T, Tagliani L, Miller M, Wang N, Pang H, Rudert M, Schroeder S, Hondred D, Seltzer J, Pierce D (2000) *Agrobacterium*-mediated sorghum transformation. *Plant Mol Biol* 44:789–798