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

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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

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 (Yamaaji 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 DA1 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 radiate (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, DA1, 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).
A. tumefaciens strain EHA 105 carrying binary vector was cultured on minimum AB medium (Chilton et al. 1974) supplemented with the following antibiotics: 100 μg/ml spectinomycin, 300 μg/ml streptomycin, and 60 μg/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 acids, and 100 μM 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 μl 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.

**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 μM 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-Infeciton | 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 μM 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 |

**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 μmol m$^{-2}$ 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'-GCACGCAACGCTACGACTGG-3'
- **Bar-Rv-3**: 5'-TCAGATCTCGTGACGCGCAG-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 digoxygenin-labeled PCR probe for bar overnight and then incubated with the alkaline phosphate conjugated anti-digoxygenin 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 % 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.
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).

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.
**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
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-80. Overall, 23 transgenic plants from eight constructs (p7i2x-\textit{AVP}1, p7i2x-\textit{Lsi}1, p7i2x-\textit{DA}1, p7i2x-\textit{Rubisco}1, p7i2x-\textit{Rubisco}2, p7i2x-\textit{TOR}, p7i2x-\textit{AtNCED}3, and p7i2x-\textit{GLU}2) 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 \textit{Streptomyces hygroscopicus} encoding the phosphinothricine 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 \textit{Streptomyces hygroscopicus} (Bayer et al. 1972) by

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### 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             | p7i2x-\textit{AVP}1 | 0.01603 | 186 |
| PPT             | p7i2x-\textit{AVP}1 | 0.03475 | 85 |
| PPT-BIA         | p7i2x-\textit{AVP}1 | 0.03333 | 89 |
| BIA             | p7i2x-\textit{AGLU}2 | 0.00493 | 607 |
| PPT             | p7i2x-\textit{AGLU}2 | 0.00402 | 745 |
| BIA             | p7i2x-\textit{AtNCED}3 | 0.01542 | 193 |
| PPT             | p7i2x-\textit{AtNCED}3 | 0.02370 | 125 |
| PPT-BIA         | p7i2x-\textit{AtNCED}3 | 0.00164 | 1829 |
| BIA             | p7i2x-\textit{D}A1 | 0.00503 | 595 |
| PPT             | p7i2x-\textit{D}A1 | 0.03659 | 81 |
| PPT-BIA         | p7i2x-\textit{D}A1 | 0.01316 | 227 |
| BIA             | p7i2x-\textit{L}si1 | 0.02841 | 104 |
| PPT             | p7i2x-\textit{L}si1 | 0.01786 | 167 |
| PPT-BIA         | p7i2x-\textit{L}si1 | 0.01427 | 209 |
| BIA             | p7i2x-\textit{PLD}-alpha | 0.05556 | 53 |
| PPT             | p7i2x-\textit{PLD}-alpha | 0.01282 | 233 |
| PPT-BIA         | p7i2x-\textit{PLD}-alpha | 0.00714 | 418 |
| BIA             | p7i2x-\textit{Rubisco}1 | 0.01786 | 167 |
| PPT             | p7i2x-\textit{Rubisco}1 | 0.02139 | 139 |
| PPT-BIA         | p7i2x-\textit{Rubisco}1 | 0.01205 | 248 |
| BIA             | p7i2x-\textit{Rubisco}2 | 0.04545 | 65 |
| PPT             | p7i2x-\textit{Rubisco}2 | 0.00778 | 384 |
| PPT             | p7i2x-\textit{TOR} | 0.04712 | 63 |
| PPT-BIA         | p7i2x-\textit{TOR} | 0.01613 | 185 |

$^a$Treatment means were compared using 95% confidence intervals
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 (Miflin 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 Enrıquez-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

| 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 | Multicopy |
|-------------------------|-----------------------------------|-------------------------------------------------|-----------------------------|--------------------------|--------|----------|----------|----------|-----------|
| p7i2x-tNCED3            | 46                                | 33                                              | 25                          | 20                       | 9      | 6        | 3        | 2        | 0         |
| p7i2x-Lsi1              | 63                                | 58                                              | 34                          | 29                       | 14     | 9        | 6        | 0        | 0         |
| p7i2x-Da1               | 20                                | 18                                              | 5                           | 11                       | 8      | 2        | 1        | 0        | 0         |
| p7i2x-AVP1              | 49                                | 38                                              | 16                          | 19                       | 11     | 4        | 2        | 1        | 1         |
| p7i2x-Rubisco1          | 27                                | 26                                              | 12                          | 10                       | 5      | 1        | 2        | 2        | 0         |
| p7i2x-AGLU2             | 7                                 | 5                                               | 3                           | 2                        | 1      | 0        | 1        | 0        | 0         |
| p7i2x-LEW2              | 35                                | 32                                              | 13                          | 15                       | 8      | 4        | 3        | 0        | 0         |
| p7i2x-PLD-alpha         | 22                                | 18                                              | 6                           | 9                        | 6      | 2        | 1        | 0        | 0         |
| p7i2x-Rubisco2          | 25                                | 20                                              | 7                           | 7                        | 3      | 3        | 0        | 0        | 0         |
| p7i2x-TOR               | 27                                | 25                                              | 22                          | 12                       | 12     | 0        | 0        | 0        | 0         |
| Total                   | 321                               | 273                                             | 143                         | 134                      | 77     | 29       | 22       | 5        | 1         |
Zheng et al. (2009). Recently, Duan et al. (2012) reported a high-throughput protocol for japonica cv. Nipponbare and Wanjing 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. 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 p712x-AtNCED3. Lane 26: plasmid p712x-PLD-alpha. Lanes 27–29: not transgenic controls. Lane 30: reaction control. Size of bands: 279 bp in constructs p712x-PLD-alpha and p712x-Rubisco1 and 242 bp in remaining plasmids. Note the size difference between plants transformed either with constructs p712x-PLD-alpha and p712x-Rubisco2 (Lanes 2, 7, 8, and 11) or remaining plasmids (Lanes 4, 5, 12–15).

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.

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

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