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Development of a non-lethal selection system by using *aadA* marker gene for efficient recovery of transgenic rice (*Oryza sativa* L.)

PhD thesis (*Abstract*)

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Abstract

The application of aminoglycoside-3''-adenyltransferase (*aadA*) gene-mediated streptomycin resistance for non-lethal selection of transgenic rice resulted in as high plant regeneration frequency under selection pressure as in the non-transformed control without selection. Since streptomycin does not kill non-transgenic cells and allows plant regeneration from them, such a selection procedure was developed that made the visual identification of transgenic calli and regenerants possible. For callus-level selection, a vital pH indicator Chlorophenol Red was applied together with streptomycin utilising the phenomenon that fast growing cell lines lower pH in the culture media. Transgenic plants were selected according to their main distinctive features; the green colour (photomixotrophic assimilation) and a more intense growth. At the same time, non-transgenic regenerants possessed bleached colour (heterotrophic assimilation) and retarded growth in the presence of streptomycin and sucrose. The final efficiency of the genetic transformation based on streptomycin resistance was found to be double than that of the transformations where the selective agent was L-phosphinothricin, and nearly three times more compared to transformations resulted in Hygromycin resistant regenerants. To the best of our knowledge, this attempt is the first in producing nuclear transformant rice plant by using a non-lethal selection strategy based on the chimaeric *aadA* gene.

Introduction

Transformation of plant cells has been achieved using chimaeric genes that confer resistance to toxic drugs such as kanamycin (Bevan et al., 1983), Hygromycin (Waldron et al., 1985), methotrexate (Herrera-Estrella et al., 1983), bleomycin (Hille et al., 1986) and L-phosphinothricin (Dekeyser et al., 1989). Expression of the chimaeric genes in the transformed cells confers the ability to survive and proliferate on the selective medium while sensitive cells die (Jones et al., 1987). That is, these drugs are lethal selective agents. However, in some plant species like rice (*Oryza sativa* L.), the application of some of these selective agents interfered with the regeneration frequency of putative transgenic plants (Hodges et al., 1991). Therefore, numerous experiments were carried out in this species to find those resistance genes and toxic drugs which have no inhibitory effect on the

regeneration of transgenic plants (Hauptmann et al., 1988; Caplan et al., 1992; Christou and Ford 1995; Li et al. 1997). The fact that the lethal selection decreases or prevents plant regeneration not only in the case of non-transgenic but in the case of transgenic cells too, turned our attention towards investigating non-lethal selection strategies. Streptomycin, unlike the drugs listed above, does not kill plant cells. Differentiation in culture of resistant mutants from the sensitive parental type is based on greening and faster growth (Jones et al., 1987). Sensitive cells respond to streptomycin by bleaching and retarded growth but no cell death (reviewed in Maliga, 1984). That is, the streptomycin is a nonlethal selective agent. Bleached streptomycin-sensitive cells, lacking photosynthetic pigments can proliferate in the presence of sucrose (Jones et al., 1987). On this basis, we hypothesised that the application of aminoglycoside-3'-adenyltransferase (*aadA*) gene-mediated streptomycin resistance in genetic transformation experiments as a visual marker can result in as high plant regeneration frequency under selection pressure as in the non-transformed control without selection. According to this hypothesis, the main distinctive features of the transgenic plants are supposed to be the green colour (photomixotrophic assimilation) and more intense growth, while the nontransgenic regenerants will possess bleached colour (heterotrophic assimilation) and retarded growth in the presence of streptomycin. At the same time, we also had to solve the problem that the streptomycin, as a non-lethal selection agent, does not allow a simple identification of transgenic calli. By utilising the phenomenon that fast growing callus cultures lower the pH in the culture media (Ryu et al., 1990), the application of a vital pH indicator Chlorophenol Red (Kramer et al., 1993) was used to screen putative transgenic calli under selection pressure. Until now, tobacco (Svab et al., 1990), oilseed rape (Schröder et al., 1994) and white clover (Larkin et al., 1996) have been transformed by using *aadA* marker gene. According to our knowledge, our attempt is the first in producing a nuclear transformant rice plant using a non-lethal selection strategy, mediated by the chimaeric aminoglycoside-3'-adenyltransferase.

Materials and methods

Plant material, surface sterilisation of spike lets, isolation of immature embryos

Japonica-type Taipei 309 rice plants were grown in greenhouse at continuous 28 °C ± 2 °C and under 12h light (10000 Lux) / 12h darkness regime. Their young spike lets (about 8-10 days post anthesis) were surface sterilised in 70% (v/v) ethanol for 1 min, then in 30% (v/v)

Domestos (Unilever Hungary Kft., Budapest) for 30 min. and washed 5 times in sterile distilled water. Subsequently immature embryos were isolated and transferred into callus induction medium.

Induction of embryogenic calli

Callus formation from immature embryos was induced on LS2.5 callus induction medium (Thompson et al., 1986) modified by Oard and Rutger (1988), containing MS salts and vitamins (Murashige and Skoog 1962), 300 mg/l casein hydrolysate, 30 g/l sucrose, 6 g/l agarose, 2 mg/l 2,4-D and 1 mg/l kinetin. The pH of the medium was adjusted to 5.8 by 1 M KOH. Explants were incubated for 4 weeks at 27 °C in permanent darkness.

Gene constructs used for transformation

Three gene constructs were used for the genetic transformation experiments that differ from each other in the used selectable marker gene only. All constructs were driven by the CaMV 35S promoter from the cauliflower mosaic virus (426 bp) which was fused to a 5' untranslated leader sequence and terminated by the nopaline synthase 3' sequence (271 bp) of *Agrobacterium tumefaciens* in pBluescript KS plasmid (Strata gene, La Jolla, CA, US). Between the promoter/leader and terminator sequences, a chimaeric *aadA* gene (Goldschmidt-Clermont et al., 1991) resulting in streptomycin/spectinomycin resistance, or a *hpt* (Hygromycin phosphotransferase) gene (Waldron et al., 1985) resulting in Hygromycin resistance, or a *bar* (bialaphos resistance) gene (de Block et al. 1987) resulting in resistance to the derivatives of L-phosphinothricin were inserted at the polylinker site of the above mentioned transformation cassette.

Gene gun-mediated genetic transformation

Delivery of plasmid DNA was carried out by the GENEBOOSTER™ GBM-7 device (Elak Ltd., Budapest, Hungary) as it was described by Jenes et al. (1996) and Öktem et al. (1999). The plasmid DNA was prepared and fixed on the surface of 1.1 µm Tungsten particles (M-10, Sylvana-GTE, Towanda, PA, USA) according to the method of Klein et al. (1987). Primary callus cultures were used as target explants. Embryogenic cells from the proliferating calli were plated on a filter paper discs on nonselective callus induction medium. Each target

was bombarded 3 times at 1100 psi nitrogen gas pressure under vacuum, at 135 mm shooting distance.

Selection and regeneration of putative transgenic plants

The bombarded primary calli were left on the callus induction medium for 3 days, then were transferred onto the same medium supplemented with proper selective agents (100 mg/l streptomycin sulphate, 10 mg/l L-phosphinothricin, 100 mg/l Hygromycin) depending on the gene construct used for transformation. During the streptomycin-based selection, half of the putative transgenic calli were selected on the basis of the colour reaction of the vital pH indicator Chlorophenol Red (pH 4.6 white - pH 5.8 orange - pH 7.0 dark red) that was filter sterilised also into the callus induction media. Fast growing embryogenic callus clumps which possessed a characteristic white zone around in the orange coloured media were multiplied by repeated subculturing (in every two weeks) during the subsequent 4-5 month selection procedure. For easier identification, digital pictures were taken of these Petri dishes and the images were manipulated using Adobe Photoshop to increase contrast. The other half of the transformed callus clumps cultured on streptomycin-containing media were not pre-selected by Chlorophenol Red, all of them were propagated during the selection procedure. Surviving explants were transferred onto modified MSD4 regeneration medium (Thompson et al., 1986) containing MS salts and vitamins, 30 g/l sucrose, 40 g/l maltose, 6 g/l agarose, 4.0 mg/l BAP, 0.5 mg/l IAA, 0.5 mg/l NAA and the proper selective agents as above. The pH of the medium was adjusted to 5.8 by 1 M KOH. Explants were incubated first for 2 weeks at 27 °C in dim light, then under 12h light (5000 Lux) / 12h darkness regime. Regenerated plantlets (20 mm in size) were transferred into sterile plastic containers (VegBox) containing hormone-free N6 medium (Chu, 1978) solidified with 3 g/l Gelrite. The next subculturing took place when plantlets reached the 70-80 mm in size. At this stage they were transferred to VegBox containers containing hormone and sugar-free N6 medium solidified also with 3 g/l Gelrite. Plantlets with well-developed root system were potted into soil under greenhouse conditions.

PCR amplification of specific DNA fragments

Total DNA samples from the greenhouse-grown, 1.5 month old wild type and putative transgenic plants were extracted using Qiagen Plant Mini Kit (Qiagen GmbH, Hilden, Germany), as well as plasmid DNA isolation from *E. coli* was done using Qiagen Plasmid

Mega Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. PCR reactions were carried out in 25 µl of commercial buffer (Eurobio, Germany) containing 1 mM MgCl₂, 200 µM dATP, dTTP, dCTP, dGTP (Pharmacia), Taq polymerase (Eurobiotaq, 3 Units/sample), DNA (100 ng total, or 10 pg plasmid), and oligonucleotide primers in 25 pmol/25 µl concentration. To amplify transformation cassette-specific fragments the following primer pairs were used:

aadA-specific fragment (800 bp): (+) 5'-GAAGCGGTTATCGCCGAAG-3'
(-) 5'-TTATTTGCCAACTACCTTAGTGATC-3'

hpt-specific fragment (375 bp): (+) 5'-GCTGGGGCGTCGGTTTCCACTATCCG-3'
(-) 5'-CGCATAACAGCGGTCATTGACTGGAGC-3'

bar-specific fragment (500 bp): (+) 5'-GGCCGACATCCGCCGTGCCA-3'
(-) 5'-GCGGTACCGGCAGGCTGAAGT-3'

PCR reactions were performed in a Perkin Elmer Cetus thermocycler (denaturation at 94 °C, 1 min; annealing at 60 °C 30-60 s; extension at 72 °C 1 min 30 s; 35 cycles). Amplification products were analysed by electrophoresis in 1.5% agarose/ethidium bromide gels.

DNA Hybridisation Experiments

The frequency of rice transformation was evaluated by Southern-hybridisations (Southern et al., 1975). PCR amplified fragments of the chimaeric marker genes were separated and isolated using gel electrophoresis, and then labelled with ³²P[ATP] random priming (Feinberg and Vogelstein, 1983). These labelled *aadA*, *hpt* and *bar* fragments were mixed in 1:1:1 proportion and then were used as a probe mixture in the DNA hybridisation experiments. Figure 3B is a digital photograph that was taken from the original filter. Prior to blotting, isolated plant and plasmid DNA were digested by *Bam*HI and *Eco*RI to cut out the three kinds of transformation cassettes. The resulted fragments were; *pCaMV35S::aadA::nos* = 1.6 kb; *pCaMV35S::hpt::nos* = 1.9 kb; *pCaMV35S::bar::nos* = 1.4 kb. Restriction endonuclease digested and depurinated DNA fragments were run in 0.8-1.5% agarose gels and transferred onto Hybond-N+ nylon membrane (Amersham Chem. Co.). Hybridisations,

cleavage of plant and plasmid DNA, and the autoradiography were carried out according to the laboratory manual of Sambrook et al. (1989).

Statistical analysis

The investigations were done in several repetitions, in most cases 3 or 5 times. This information is shown in each case in the appropriate section of 'Results' or in the legend of the corresponding tables. The data were evaluated by mean analysis, and standard deviations were calculated from weighted differences of the mean (Sváb, 1981). Significant differences were stated where data combined with their standard errors had no overlaps.

Results

First the impacts of the three corresponding selection agents (streptomycin, hygromycin, L-phosphinothricin) were examined on the morphogenesis of non-transformed cell cultures of rice (Table 1). The most important difference among the selective agents was that streptomycin did not suppress the callus growth and plant regeneration significantly, compared to the control, while both hygromycin and L-phosphinothricin induced an immediate stop in callus development and a dramatic cell death subsequently. In the case of streptomycin, a gradual slow down could be obtained in the callus growth (data not shown) that was directly proportional to the increasing concentrations of the antibiotic. This moderate effect of the streptomycin was detected also during the plant regeneration phase. It showed no inhibition of plant regeneration at the most concentrations applied (10, 25, 50, 100 mg/L), a slight decrease was obtained only at 150 mg/L. However, instead of influencing the morphogenetic responses, the increasing streptomycin concentrations resulted in a gradually elevated proportion of bleached regenerants. As a result of this process, plantlets regenerated on 100 mg/L streptomycin containing media were all bleached (Figure 1A). These plants were sensitive to photooxidative processes and were non-viable in the absence of a sugar source. The above results provided a very promising tool into our hands in terms of visual selectability. After the above optimisations, gene gun-mediated transformation experiments were carried out. The subsequent selection procedure was done as previously shown in Table 1. For this purpose 100 mg/L streptomycin, 100 mg/L hygromycin and 10 mg/L L-phosphinothricin were used. Streptomycin as a non-lethal selection agent did not make the detection of calli consisting of mainly transgenic cell lines possible. Therefore a technically

simple and fast technique has been developed for this purpose by the application of a vital pH indicator Chlorophenol Red during the callus-level selection. As a result, we could identify those calli satisfactorily from which transgenic regenerants could be recovered (Figure 2A). For an easier identification, digital pictures were taken about these Petri dishes that were manipulated by Adobe Photoshop program for increased contrast (Figure 2B). Blue areas represented the originally yellow ones where the pH remained unchanged (nt), while black areas indicated putative transgenic calli where the pH of the culture media decreased dramatically (pt).

All the putative transgenic plants – 27 plants transformed with *aadA* gene, 11 plants transformed with *hpt* gene and 16 plants transformed with *bar* gene - were subjected to PCR tests and Southern-hybridisations in order to confirm the presence of transgenes in their nuclear genomes. Results of three putative transformants carrying one given gene construct were displayed in Figure 3A and B.

Primers designed into the *aadA*, *hpt* and *bar* genes gave clear bands in the transformed rice lines (Figure 3A) that were chosen for demonstration (*aadA* construct: lanes Ta1, Ta2, Ta3, *hpt* construct: Th1, Th2, Th3, *bar* construct: Tb1, Tb2, Tb3) and in the positive controls (lanes Ca, Ch, Cb) prepared from the corresponding gene constructs. However, no products from non-transformed rice plants (lane Wt) and from transformed white regenerants (lane Tw) could be amplified with these primers.

Southern hybridisations (Figure 3B) strengthen the results of PCR analysis showing that transformation cassettes were integrated into the genome in transformed green regenerants in the majority of cases (*aadA* construct: lanes Ta1, Ta2, Ta3, *hpt* construct: Th1, Th2, Th3, *bar* construct: Tb1, Tb2, Tb3). Positive controls (purified DNA from plasmids applied for transformation) also gave strong hybridisation signals (lanes Ca, Ch, Cb), while no homologous fragment was present in the wild type control plants (lane Wt) and in the transformed white regenerants (lane Tw). Common feature of the transgenic lines was a frequent rearrangement of transgene copies (in 30-60% of transformation events) that is typical of biolistic transformation (Figure 3 B: lanes Ta1, Th2, Tb1, Tb3).

To conclude, the majority of transformed plants (70-100%) that were regenerated in the presence of the proper concentrations of hygromycin or L-phosphinothricin were proved to be transgenic, while in the case of streptomycin, green plantlets (Figure 1B), represented approximately the 30% of regenerants, carried the chimaeric genes only (Table 2). However, by using Chlorophenol Red pre-selected streptomycin resistant calli for plant regeneration, the laborious and costly culturing of non-transgenic plant material could be avoided. After all, the

final efficiency of the streptomycin-based selection was found to be double than that of the L-phosphinothricin-based one and nearly three times more than that of the selection based on hygromycin.

Discussion

A number of selective agents and suitable resistance genes have been investigated in rice concurrently with the studies on gene transfer and cell culture (Christou and Ford, 1995). The most widely used lethal inhibitors in this species are G418, hygromycin, and derivatives of phosphinothricin (Dekeyser et al., 1989). The first two are aminoglycoside antibiotics, which interfere with the translation machinery of prokaryotic and eukaryotic cells (Herrera-Estrella et al., 1983), while phosphinothricin, a fungal amino acid, inhibits nitrogen assimilation in higher plants (Thompson and Seto, 1995). All the lethal selection agents listed above have advantageous and disadvantageous features from the point of view of genetic transformation that differ species to species even, sometimes, genotypes to genotypes within a species. Disadvantageous features of a selective agent can be divided to four categories as follows. First, when a given plant species has natural tolerance to the inhibitor. Gramineous plants, for example, show a high G418 tolerance, making it very difficult to separate transformed from non-transformed tissues with chimaeric *npt-II* gene constructions (Hauptmann et al., 1988). Second, when a selection agent is a competitive inhibitor. As for example phosphinothricin (PPT) is a competitive inhibitor of glutamine synthetase (GS) that plays a central role in the assimilation of inorganic nitrogen. PPT impedes the binding of glutamic acid (a natural substrate of GS) to the active centrum of the enzyme, and in this way, it results in toxic accumulation of ammonia in the cytoplasm (Thompson and Seto, 1995). However, the competitive character of PPT also means that by supplying exogenous glutamic acid or by overproduction of GS, its inhibitory effects can be repressed (D'Halluin et al., 1992; Thompson and Seto, 1995). Since the endogenous level of glutamic acid depends on the physiological condition and morphogenetic phase of a plant explant, the optimal concentration of PPT varies during the selection procedure (Toldi et al., 2000 a.; b.). Third, when an inhibitor has a non-specific side-effect on the metabolism of plant cells. An example is that aminoglycoside antibiotics are inactivated by phosphorylation reactions mediated by the products of either the Tn5 neomycin phosphotransferase (*npt-II*) gene or by hygromycin phosphotransferase (*hpt*) gene from *Escherichia coli*. The energy source and the phosphate donor of this reaction is the ATP reservoir of the transformed cell, which means that part of

the accumulated chemical energy utilised to inactivate the inhibitor, instead of promoting essential metabolic and morphogenetic processes. Last but not at least, the fourth category corresponds to those cases when a selective agent possesses hormone-like activity, which interferes with the regeneration of putative transgenic plants. The example is again PPT that has a cytokinin-like morphogenetic effect in snapdragon (Hoshino and Mii, 1998), in rice (Toldi et al., 1999; 2000 a.; b.) and in tobacco (Szegedi et al., personal communication) making the re-optimisation of tissue culture process necessary in its presence. These disadvantageous features of lethal selection strategies lead us to look for an alternative procedure, in which transgenic plants could be selected by such agents that cause only virtual symptoms, but not severe tissue toxicosis. On the other hand, we also wanted to avoid the application of such marker genes that allow visual selection, but do not support the faster growth of transgenic cell lines, at the same time. An important precondition of a successful selection procedure is that transgenic cell lines must overgrow non-transgenic ones by its end (Jones et al., 1987). On this basis our aim was the establishment of a new selection method in rice by combining advantageous features of the lethal and non-lethal strategies. We planned the development of such procedure in which the rapid growth of transgenic cell lines is supported without causing cell necrosis in non-transgenic ones during the callus phase. Plant regeneration, however, can occur in this system not only from transgenic cells, but from non-transgenic ones too. Therefore, we also had to find a way to distinguish between transgenic and non-transgenic calli and regenerated plants in a non-invasive manner.

For the above purposes, the aminoglycoside-3"-adenyltransferase (*aadA*) gene mediated streptomycin resistance was chosen. Experiments were designed so that parameters of our selection protocol were evaluated together with the two most frequently used alternatives, the hygromycin resistance gene (*hpt*) based and the L-phosphinothricin resistance gene (*bar*) based selections, comprehensively. First, a simple procedure was developed for callus-level selection that allowed a fast visual (Figure 2A) and computer aided video image analysis (Figure 2B). Since the high activity of membrane linked proton-pump ATPases in fast growing callus clumps lowers the pH in the surrounding media dramatically (Ryu et al., 1990; Michelet and Boutry, 1995), the use of a vital pH indicator Chlorophenol Red (Kramer et al., 1993) made the identification of transgenic calli possible in the desired way (Figure 2A, B, Table 2). The majority of transformed plants that were regenerated in the presence of the proper concentrations of hygromycin (100%), L-phosphinothricin (70%) and streptomycin + Chlorophenol Red (60%) were proved to be transgenic (Table 2), while in the case of streptomycin, green plantlets (Figure 1B), represented approximately the 30% of regenerants,

carried the chimaeric genes only. Despite of this, the final efficiencies of the streptomycin and streptomycin + Chlorophenol Red based selections were found to be double than that of the L-phosphinothricin-based one and nearly three times more than that of the selection based on hygromycin.

The application of aminoglycoside-3'-adenyltransferase (*aadA*) gene-mediated streptomycin resistance for non-lethal selection of transgenic rice resulted in as high plant regeneration frequency under selection pressure as in the non-transformed control without selection. The main distinctive features of the transgenic plants were the green colour (photomixotrophic assimilation) and a more intense growth, while the non-transgenic regenerants possessed a bleached colour (heterotrophic assimilation) and retarded growth in the presence of streptomycin and a sugar source.

To the best of our knowledge, this attempt is the first in producing nuclear transformant rice plant by using a non-lethal selection strategy based on the chimaeric *aadA* gene.

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