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Plastid Transformation in the Monocotyledonous Cereal Crop, Rice (*Oryza sativa*) and Transmission of Transgenes to Their Progeny

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Abstract

The plastid transformation approach offers a number of unique advantages, including high-level transgene expression, multi-gene engineering, transgene containment, and a lack of gene silencing and position effects. The extension of plastid transformation technology to monocotyledonous cereal crops, including rice, bears great promise for the improvement of agronomic traits, and the efficient production of pharmaceutical or nutritional enhancement. Here, we report a promising step towards stable plastid transformation in rice. We produced fertile transplastomic rice plants and demonstrated transmission of the plastid-expressed green fluorescent protein (GFP) and aminoglycoside 3′-adenylyltransferase genes to the progeny of these plants. Transgenic chloroplasts were determined to have stably expressed the GFP, which was confirmed by both confocal microscopy and Western blot analyses. Although the produced rice plastid transformants were found to be heteroplastomic, and the transformation efficiency requires further improvement, this study has established a variety of parameters for the use of plastid transformation technology in cereal crops.

Keywords

Cereal Crop; Chloroplast Genome; Mono-cotyledonous Plant Species; Plastid Transformation; Rice

Introduction

The plastid transformation approach has been shown to have a number of advantages, most notably with regard to its high transgene expression levels (De Cosa *et al*., 2001; Devine and Daniell, 2004), capacity for multi-gene engineering in a single transformation event (Daniell and Dhingra, 2002; De Cosa *et al*., 2001; Lossl *et al*., 2003; Quesada-Vargas *et al*., 2005; Ruiz *et al*., 2003) and ability to accomplish transgene containment via maternal inheritance (Daniell, 2002; Daniell *et al*., 1998; Svab and Maliga, 1993). Moreover, chloroplast appears to be an ideal compartment for the accumulation of certain proteins, or their biosynthetic products, which would be harmful if they accumulated in the cytoplasm (Daniell *et al*., 2001; 2005a; Lee *et al*., 2003; Leelavathi and Reddy, 2003; Ruiz and Daniell, 2005). In addition, no gene silencing has been observed in association with this technique, whether at

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the transcriptional or translational level (DeCosa *et al*., 2001; Dhingra *et al*., 2004; Lee *et al*., 2003). Because of these advantages, the chloroplast genome has been engineered to confer several useful agronomic traits, including herbicide resistance (Daniell *et al*., 1998; Kang *et al*., 2003), insect resistance (Kota *et al*., 1999; McBride *et al*., 1995), disease resistance (DeGray *et al*., 2001), drought tolerance (Lee *et al*., 2003), salt tolerance (Kumar *et al*., 2004a), and phytoremediative ability (Ruiz *et al*., 2003). The chloroplast genome has also been utilized in the field of molecular farming, for the expression of biomaterials, human therapeutic proteins, and vaccines for use in humans or animals (Fernandez-San Milan *et al*., 2003; Guda *et al*., 2000; Koya *et al*., 2005; Leelavathi *et al*., 2003; Molina *et al*., 2004; 2005; Staub *et al*., 2000; Vitanen *et al*., 2004; Watson *et al*., 2004; for review, see Bock, 2001; Daniell *et al*., 2004a; 2004b; 2005a; Grevich and Daniell, 2005; Maliga, 2004).

Although plastid transformation has been accomplished via organogenesis in a number of dicotyledonous plant species, including tobacco (Svab and Maliga, 1993), tomato (Ruf *et al*., 2001), potato (Nguyen *et al*., 2005; Sidorov *et al*., 1999), *Arabidopsis* (Sikdar *et al*., 1998), *Lesquerella* (Skarjinskaia *et al*., 2003), oilseed rape (Hou *et al*., 2003), petunia (Zubko *et al*., 2004) and lettuce (Lelivelt *et al*., 2005), this technology has proven to be highly efficient only in tobacco. In the *Arabidopsis*, 1 transplastomic line per 40 or 151 bombarded plates was obtained, but these lines were sterile. In the potato, 1 transplastomic line was obtained per 18 or 25 bombarded plates, but no data was reported with regard to the fertility of these lines. In the tomato and petunia, 1 transplastomic line was obtained per 10 bombarded plates. However, in the tobacco, up to 14 transplastomic lines were obtained per bombarded leaf (Daniell *et al*., 2001a). In the case of *Lesquella*, transplastomic clones had to be grafted onto *Brassica napus* rootstock in order to obtain seeds. Plastid transformation of oilseed rape produced heteroplastomic plants, and no data was reported regarding the fertility of these lines. The aforementioned studies have all reported attempts to accomplish plastid transformation in dicotyledonous plant species, and have apparently met with varying degrees of success.

Major obstacles to the extension of plastid transformation technology to crop plants which regenerate via somatic embryogenesis include: (i) the difficulty of expressing transgenes in non-green plastids, in which gene expression and gene regulation systems are quite distinct from those of mature green chloroplasts, and (ii) our current inability to generate homoplastomic plants via subsequent rounds of regeneration, using leaves as explants. Despite these aforementioned limitations, plastid transformation has recently been accomplished in several primary dicotyledonous crops, including soybean, carrot, and cotton, via somatic embryogenesis (Dufourmantel *et al*., 2004; 2005; Kumar *et al*., 2004a; 2004b). Breakthroughs in the plastid transformation of recalcitrant crops, such as cotton and soybean, have raised the possibility of engineering plastid genomes of other major crops, via somatic embryogenesis. However, despite the great interest to extend plastid transformation to major cereal crops, there has not yet been even a single report of a fertile transplastomic plant which verifiably transmitted transgenes to the next generation in any monocotyledonous plant species. To date, only a couple of fragmentary data were reported in association with rice (Khan and Maliga, 1999). However, the purpose of the study was to test the feasibility of using a fluorescent antibiotic resistance marker for tracking of plastid transformation in higher plants, including rice suspension cells, not to test the feasibility of plastid transformation in rice itself.

Here, we report a promising step towards stable plastid transformation in rice. The transplastomic rice plants generated in this study exhibited stable integration and expression of the *aadA* and *sgfp* transgenes in their plastids. Moreover, the transplastomic rice plants generated viable seeds, which were confirmed to transmit the transgenes to the T1 progeny plants. However, it was impossible to convert the transplastomic rice plants to homoplasmy,

even after two generations of continuous selection. Potential limiting factors in the routine application of plastid transformation to rice are discussed in this article, as are some possible solutions to these problems.

Materials and Methods

Construction of pLD-RCtV-sGFP

A rice-specific plastid transformation vector, pLD-RCtV-sGFP, targets insertions into the *trnI-trnA* inverted repeat regions of the rice plastid genome (at nucleotides 93072-94942 and 120176-122046; GenBank accession No. NC_001320). The *trnI-trnA* region was amplified from the rice plastid genome by PCR, using the primers, OS6P (anneals to nucleotides 92731-92755 and 122363-122387) and OS6M (anneals to 94919-94942 and 120176-120199). The 2,212 bp PCR product was then cloned into pCR2.1-TOPO (Invitrogen, USA). The *Stu*I/*Eco*RV 1.89 kb fragment was inserted into the *Pvu*II site of pUC19. The selection marker gene, *aadA*, which is driven by the 16S ribosomal RNA promoter (Prrn), chloroplast ribosome binding site (GGAGG), and the 3′ UTR of the plastid *psbA* gene were excised as a *Not*I-*Not*I fragment, from a basic chloroplast transformation vector which had been constructed in the Daniell lab. The ends were then filled in with Klenow enzyme in order to clone them into the unique *Pvu*II site located between the *trnI* and *trnA* regions (94117-94122 or 120996-121001), to obtain pLD-RCtV. The *sgfp* gene sequence (Chiu *et al.*, 1996) was then amplified by PCR, using the pSK-RG plasmid (Jang *et al.*, 2002) as a template, as well as primers designed to introduce a ribosome binding site, which was to be located upstream of the start codon and the *Xba*I flanking sites. The PCR-amplified fragment was then cloned into the *Xba*I site, which was located between the *aadA* gene and the 3′ UTR of *psbA* of pLD-RCtV, resulting in the generation of pLD-RCtV-sGFP.

Transformation and regeneration

We have previously optimized the conditions for the efficient regeneration of plants from the japonica rice (*Oryza sativa* L.) variety, Hwa-Chung (Lee *et al.*, 2002), and closely followed the protocols for the regeneration of transplastomic plants. Mature seeds were positioned on N6 medium (Chu *et al.*, 1975), which had been supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) (2 mg/L), casein hydrolysate (1 g/L) and sucrose (30 g/L), and had been semi-solidified by the addition of 0.2% (w/v) gelrite (Duchefa, Haarlem, Netherlands), in order to elicit callus formation. After 15–20 days of seed-planting, the total calli, both embryogenic and non-embryogenic (Lee *et al.*, 2002; Wang *et al.*, 2004), were collected in the central region of the regeneration medium, which contained Murachige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962), kinetin (2 mg/L), 1-naphtaleneacetic acid (NAA) (2 mg/L), myo-inositol (100 mg/L), sucrose (30 g/L), and gelrite (2 g/L). The total calli were then bombarded with the pLD-RCtV-sGFP plasmid DNA-coated gold particles of 0.6 μm diameter, using a PDS-1000/He Biolistic gun device and 1,100 p.s.i. rupture disks (Bio-Rad, USA). After bombardment, the calli were incubated in darkness at 27°C for 1–2 d prior to transfer into a selective regeneration medium supplemented with 200 mg/L of streptomycin sulfate (Sigma, USA) and 14 g/L of phytagar (Gibco-BRL, USA). In an additional selection step, the streptomycin-resistant calli which produced shoots were transferred to fresh selective regeneration medium supplemented with 300 mg/L of streptomycin. The streptomycin-resistant shoots were then rooted on MS medium which contained MS salts, MS vitamins, sucrose (30 g/L), and gelrite (2 g/L), supplemented with 500 mg/L of streptomycin. The plantlets were maintained in the selective MS medium for 2–3 months, then transplanted into pots filled with soil.

In order to detect functional sGFP expression in the T1 progeny, the seeds from the self-pollinated T0 transplastomic plants were germinated, then allowed to grow for 8 weeks on MS medium supplemented with 100 mg/L of streptomycin. The progeny seedlings were then transferred to fresh MS selective medium supplemented with 300–500 mg/L of streptomycin, and maintained for 3 months before being transferred to a greenhouse.

PCR analyses

Total genomic DNA was extracted from the leaf tissues of both the transformed and untransformed plants, according to the methods described by Doyle and Doyle (1987). PCR analysis was conducted using Excel Taq DNA polymerase (Corebio, Korea), using 100 ng genomic DNA as a template. For the PCR analyses of the T0 transplastomic plants, we amplified the *sgfp* gene with the forward primer (5′-GACCCTGAAGTT-CATCTGAC-3′) and the reverse primer (5′-ACTTGTACAGCT CGTCCATG-3′). The left border fragment was amplified with the forward primer (5′-TCAGCCATACGGCGGTGAATCCG-3′) and the reverse primer (5′-CCGCG-TTGTTTCATCAAG-CCTTACG-3′). The right border fragment was amplified with the forward primer (5′-CGGGATCACTCACGGCATGGACG-AG-3′) and the reverse primer (5′-TACCATAGAGGCCAACG-ATAGACAATAA-3′). The left and right border PCR products were cloned into pGEM-T Easy vector (Promega, USA) for DNA sequencing. For the PCR analysis of the T1 progeny plants, a set of 3 primers, trnI-F (5′-TGATTCTCTCCCAATTGGTT-GGATCGTA-3′), aadA-R (5′-CGTCGTGCACAACAATGGTG-ACTTCTAC-3′) and trnA-R (5′-CAATTAGACAGCCAACCC-3′), at a ratio of 1:1:0.05 (trnI-F: aadA-R: trnA-R), was used to detect the level of transplastome relative to untransformed plastome. The primer locations are all shown in Fig. 1.

Southern analysis

Samples representing 18 μg of the *Bam*HI-cut total genomic DNA were separated on 0.7% agarose gel, then transferred to Hybond-N+ nylon membranes (Amersham, Germany). These blots were hybridized with *aadA*-specific probe labeled with [α-^{32}P]dCTP (Amersham, Germany). The probe was generated by PCR, using the following set of primers: aadAP1 (5′-GATCGCCGAAGTATCGACTC-3′) and aadAP2 (5′-ATTTGCCGACTACCTTGGTG-3′). The membrane was autoradiographed using X-ray film.

Western analysis

Total soluble proteins were extracted from the leaves of the transformed and untransformed plants in a greenhouse, using protein extraction buffer (0.1 M Tris-HCl pH 8.3, 0.5 M NaCl, 5 mM dithiothreitol, 5 mM EDTA, 2 mM phenyl-methylsulfonylfluoride). Samples which represented 2 μg of total soluble proteins were then electrophoresed on 12% SDS-PAGE gel, and transferred to nitrocellulose membranes (Amersham, Germany) for immunoblotting. Monoclonal anti-GFP (Sigma) was used as a primary antibody at a dilution of 1:500, and we used alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, USA) as a secondary antibody, at a dilution of 1:10,000. The BCIP/NBT Combo (Gibco-BRL, USA) was used for detection.

Detection of GFP fluorescence by confocal laser scanning microscope

Fluorescence was observed in the T1 progeny of the plastid transformants with a Carl Zeiss 510 CLSM (Jena, Germany). The laser was operated at an excitation wavelength of 488 nm and an emission wavelength of 510 nm. All images were then viewed and processed with

Zeiss LSM Image Browser software. At 2 weeks after germination, leaf tissues from T1 progeny seedlings were used as samples for the confocal microscopic analysis.

Results

Construction of rice plastid transformation vector

The rice-specific plastid transformation vector, pLD-RCtV-sGFP (Fig. 1), contains the *sgfp* as a reporter gene, which encodes a synthetic GFP (Chiu *et al*., 1996; Hass *et al*., 1996), and the *aadA* gene, which confers streptomycin resistance for transformant selection (Goldschmidt-Clermont, 1991). Unlike the insoluble wild-type *gfp* gene product (Haseloff *et al*., 1997), the protein encoded by *sgfp* gene has been previously shown to exhibit no cytotoxicity in the rice plants (Jang *et al*., 2002). Both of the genes are driven by the strong plastid rRNA promoter, Prrn (Rapp *et al*., 1992), which was obtained from tobacco, and both employ the 3′ untranslated region (UTR) of the *psbA* gene for the stabilization of the mRNA (Stern and Gruissem, 1987). The capacity of the plastid for dicistronic expression of the *aadA* and *gfp* genes has been previously shown (Jeong *et al*., 2004). The suitability of the tobacco Prrn for use in transgenic expression in embryogenic cells and leaves of rice has also been demonstrated (Khan and Maliga, 1999; Silhavy and Maliga, 1998). The *trnI-trnA* intergenic region, located in the inverted repeat region of the plastid genomes, has been extensively exploited as the homologous recombination sequences for plastid transformation in tobacco, as well as in other crop species (Devine and Daniell, 2004; Dhingra *et al*., 2004). Hence, the *trnI-trnA* intergenic region was selected for plastid transformation in rice. The coding regions in the *trnI-trnA* genes are highly conserved among the higher plants (Hiratsuka *et al*., 1989; Koch *et al*., 1981; Michel *et al*., 1989), whereas introns in the *trnI-trnA* genes are not well conserved. For example, the introns in this region of rice and tobacco differ in 1 large and 5 small insertions and deletions and 25 point mutations. Therefore, the *trnI-trnA* flanking sequence in pLD-RCtV-sGFP was derived from the rice plastid genome. This flanking sequence also contains the *ori*A sequence (located within the *trnI* gene), and this is believed to enhance transgene integration (Kumar *et al*., 2004a; 2004b).

Evaluation of streptomycin selection conditions for rice plastid transformants

The *aadA* gene, which inactivates spectinomycin and streptomycin, is the most extensively used selection marker in the process of plastid transformation (Goldschmidt-Clermont, 1991; Svab and Maliga, 1993). The majority of homoplastomic plants produced thus far have been selected, under light conditions, and using spectinomycin (Ruf *et al*., 2001; Sidorov *et al*., 1999; Svab and Maliga, 1993). However, most cereal crops, including rice, exhibit natural spectinomycin resistance, due to the presence of point mutations in their 16S rRNAs sequence, which prevents spectinomycin binding (Fromm *et al*., 1987). Therefore, streptomycin resistance was employed in the selection of plastid transformants in rice. In order to determine the optimal streptomycin levels for use in the selection of rice plastid transformants, the total calli obtained 2–3 weeks after germination, containing both embryogenic and non-embryogenic ones, were positioned on shoot regeneration medium supplemented with a variety of streptomycin concentrations. Under light conditions, callus growth was partially inhibited at 100 mg/L of streptomycin, and no shoots developed, even after 2 months of culture. At streptomycin levels of between 200–500 mg/L, callus growth was inhibited to a greater extent (Fig. 2A). At 1,000 mg/L of streptomycin, all of the calli turned brown within 3 weeks of culture (data not shown), revealing that the selection had been too stringent. However, when selection progressed in darkness, callus growth did not appear to be significantly affected by the presence of streptomycin levels up to 1,000 mg/L, after 1 month of culture (Fig. 2B). We observed only a moderate degree of growth inhibition at streptomycin levels of more than 1,000 mg/L (data not shown). This suggests that the

calli, when grown in darkness, are less sensitive to streptomycin. Therefore, the selection of transformants in darkness would be less efficient than transformant selection in the light. Therefore, in the following plastid transformation experiments, we conducted transformant selection in the light, using 200 mg/L of streptomycin as the optimal concentration for the initial selection phase. For the subsequent rounds of selection, we used 300–500 mg/L of streptomycin.

Plastid transformation and recovery of transplastomic plants

Plastid transformation was conducted by the bombardment of the total calli with pLD-RCtV-sGFP. After the bombardment, the total calli were incubated in the light, on shoot regeneration medium which contained 200 mg/L of streptomycin. The streptomycin-resistant calli exhibited green spots after one month of growth on the selective medium, and shoots emerged from the calli after another month (Fig. 3A). During the second round of selection, the shoot-producing streptomycin-resistant calli were transferred to a fresh selective medium, containing 300 mg/L of streptomycin, and were maintained for a month. The streptomycin-resistant shoots were then rooted in 500 mg/L of streptomycin, and were maintained for 2–3 months on the selection medium. In 10 independent transformation experiments, in which approximately 4,000 total calli were employed (on 100 to 120 bombarded plates), we selected two independent lines, and these were regenerated into plantlets (Fig. 3B). These two streptomycin-resistant lines were designated as sGFP-1 and sGFP-2, respectively.

The putative transformants were then transferred into pots filled with soil, and grown to maturity in a greenhouse. In order to ensure continuous selection pressure, we added 500 mg of streptomycin into one liter of the soil per week. When sufficient numbers of tillers had appeared from sGFP-1 and sGFP-2, the tillers of sGFP-1 and sGFP-2 were divided into 3 separate parts, and transplanted into 3 different soil-containing pots. These tillers were then treated weekly with 500, 1,000, and 1,500 mg/L of streptomycin, respectively. In the presence of 500 mg/L of streptomycin, untransformed plants exhibited moderately inhibited growth, whereas the growth of the sGFP-1 and sGFP-2 plants appeared normal, and both of these lines produced viable seeds for T1 progeny analyses (Fig. 3C). At 1,000 mg/L of streptomycin, the vegetative growth of sGFP-1 and sGFP-2 was not affected significantly, but both lines were found to have produced either no, or few viable seeds. With a selection pressure exerted by 1,500 mg/L of streptomycin, both sGFP-1 and sGFP-2 exhibited significantly inhibited growth, and neither lines produced viable seeds.

The putative transformants were initially tested by PCR screening for the presence of the *sgfp* gene, using total genomic DNA. All six of the plants derived from sGFP-1 and sGFP-2, which had been selected with 500–1,500 mg/L of streptomycin in the greenhouse, were found to harbor 590 bp *sgfp* fragments (Fig. 4A). This indicates that streptomycin-resistant phenotype constitutes a reliable indicator for the determination of transformants. Further molecular analyses were conducted with sGFP-1 and sGFP-2 lines grown on 500 mg/L of streptomycin, which generated viable seeds for T1 progeny analyses. Southern analysis using the *aadA* probe detected a 3.3 kb fragment in the *Bam* HI-digested genomic DNA (Fig. 4B), further verifying the integration of the *aadA* gene in the transformants. Then, in order to confirm the site-specific integration of the *sgfp* and *aadA* genes into the plastid genome, sGFP-1 and sGFP-2 were subjected to PCR analysis, using one internal primer which binds to the *sgfp* or *aadA* gene, and a second primer, which binds to the native plastid genome adjacent to the flanking sequences utilized in homologous recombination (Fig. 1). Both of the lines generated both the left and right border DNA fragments of 1.9 and 1.3 kb in size, respectively, as was expected with the transplastomes (Fig. 4C). The identity of these amplified PCR products was confirmed via DNA sequencing, thus eliminating the possible attribution of the amplified DNA fragments to a PCR artifact. We also tested

sGFP-1 and sGFP-2 plants for the expression of sGFP, via immunoblotting using total soluble leaf proteins and GFP antibody. Both of the transplastomic plants produced the expected band of 27 kDa, indicating the stable expression and accumulation of sGFP in the transplastomic plants (Fig. 4D). These transplastomic plants were grown under continuous streptomycin selection conditions, and the seeds were harvested for subsequent T1 progeny analyses.

T1 progeny analyses

For dicotyledonous plant species that regenerate via organogenesis, homoplasmy can be accomplished by multiple cycles of regeneration from transformed leaf tissues under the selective pressures. However, this process of shoot subculturing is not applicable for the elimination of untransformed plastomes from rice plants. For this reason, we expected our primary transplastomic lines might be heteroplastomic. Therefore, we applied further selection pressure to the sGFP-1 and sGFP-2 progeny. All of the seeds from the self-pollinated sGFP-1 and sGFP-2 were germinated on a selective medium that contained 100 mg/L of streptomycin. A small portion of the progeny seedlings bleached, after being transferred to a selective medium that contained 300–500 mg/L of streptomycin. In contrast, only some of the seeds from the untransformed control plants were germinated in the presence of 100 mg/L of streptomycin, but these green seedlings were ultimately bleached on 300–500 mg/L of streptomycin (data not shown). After another 3 months of culturing, the streptomycin-resistant progeny plants were transplanted into soil that contained 500 mg/L of streptomycin, and grown to maturity under continuous selection pressure.

At 2 weeks after germination, the progeny seedlings were subjected to confocal laser scanning microscopic analysis, in order to examine the transgene transmission and the proportion of the transplastome in the progeny. Confocal microscopic analysis confirmed that the expression of sGFP was limited to only a small fraction of the chloroplasts (marked with arrows in Fig. 5A), in some of the T1 progeny plants. Due to the heteroplastomic state of the T0 plastid transformants, 100% transmission of the transgenes was not expected (Skarjinskaia *et al.*, 2003). Nevertheless, the localization of sGFP fluorescence in the transgenic chloroplasts constituted convincing evidence that both sGFP-1 and sGFP-2 were, in fact, plastid transformants, and that fully functional transgenes had been transmitted to the T1 progeny.

PCR analysis was also conducted with approximately 6 months-old transplastomic plants, in order to determine the levels of heteroplasmy inherent to the mature progeny. To enhance the detection limit of the transplastome and to allow approximate comparisons of the levels between the transplastomes and untransformed plastomes, PCR analysis was performed using a set of 3 primers, trnI-F, trnA-R and aadA-R, hybridizing to sequences of the *trnI-trnA* regions and the *aadA* gene (Fig. 1). Both fragments of 538 and 221 bp, corresponding to the transplastome and untransformed plastome, respectively, were detectable at different levels in the T1 progeny plants (Fig. 5B), when approximately 20-fold higher amount of the transgene-specific primer was used, than that for the rice plastid gene, *trnA* (the primer ratio of trnI-F: aadA-R: trnA-R = 1:1:0.05). This primer ratio was necessary due to the low level of the transplastomes relative to the untransformed plastomes and the bias of the DNA polymerase towards shorter amplification products in PCR reactions. Although this PCR analysis could not reveal the exact levels of heteroplasmy, differences in the intensities of the transplastomic DNA bands are likely to reflect differences in the levels of transplastomes among the individual T1 progeny plants. This result, along with the data from the microscopic analysis, indicated that the proportion of transplastomes relative to the untransformed plastomes was low, even after two generations of continuous selection.

Discussion

Rice is the primary staple crop for more than one-third of the global population. Khan and Maliga previously reported plastid transformation of rice (1999), demonstrating stable transgene integration in the rice plastome. However, the transmission of this transgene to subsequent generations could not be achieved. In the present study, we generated fertile transplastomic rice plants, in which the *aadA* and *sgfp* genes were stably integrated and expressed. Furthermore, for the first time, we have demonstrated inheritance of plastid-expressed transgenes to the progeny of a transplastomic cereal crop, although low transformation efficiency and heteroplasmy remain to be solved.

Rice plastid transformation is quite inefficient, compared to dicotyledonous plant species. There are several possible reasons for this relatively low transformation efficiency. First, efficient subculture systems with sustained plant regeneration capability for transplastomic plants are lacking in monocotyledonous cereal crops, including rice. Second, undeveloped plastids (proplastids) are used as the transformation target, rather than chloroplasts. Our transmission electron microscopic observations showed that plastids in the dark-grown calli of rice were undeveloped and approximately 1 μm in size (data not shown). This is approximately 5-fold smaller than well-developed chloroplasts in the green leaf tissue. Hence, the amount of cells exhibiting irreversible physical damage due to biolistic bombardment with 0.6 μm gold particles might be greater in the proplastids. In fact, recent work by Langbecker *et al.* (2004) indicated that the use of smaller particle sizes (0.4 μm) resulted in 3- to 4-fold increase in plastid transformation frequency during the transformation of proplastids in dark-grown tobacco suspension cells.

In addition, transcription and translation levels are lower in proplastids than in chloroplasts (Mullet, 1993; Silhavy and Maliga, 1998). This results in lower levels of selection marker gene expression to confer resistance to selective chemicals. Therefore, the recovery of the newly transformed cells is attenuated further during the initial selection phase. The P*rrn* plastid-encoded RNA polymerase (PEP) promoter was used to drive transgene expression in the rice plants. Although the Prrn promoter has been shown to be active in both the proplastids and chloroplasts of rice, its activity has been shown to be 7-fold lower in the proplastids than in the chloroplasts (Silhavy and Maliga, 1998). In fact, the proplastids are a plastid type in which most PEP promoters are either weak, or completely inactive (Silhavy and Maliga, 1998; Vera and Sugiura, 1995). While the P*rrn* nuclear-encoded RNA polymerase (NEP) promoter has been demonstrated to be active in tobacco cell suspensions and carrot cultures, it was transcriptionally silent in rice cell suspension cultures (Silhavy and Maliga, 1998). Therefore, it is unlikely that the use of full-length 16S rRNA promoter, encompassing both the PEP and NEP promoters, would increase the transgene transcription levels in the proplastids of rice. Thus far, no other promoters that accumulate high transcript levels in both the proplastids and chloroplasts of rice are currently available. As the choice of promoter appears to be restricted to P*rrn*, current efforts are likely to focus on the augmentation of protein accumulation levels in the proplastids by employing a variety of 5′ UTRs or 5′ translational control regions, which are free from light and developmental regulation (Dhingra *et al.*, 2004; Eibl *et al.*, 1999; Kumar *et al.*, 2004a; Maliga, 2003).

The other major difficulty in rice plastid transformation involves the acquisition of homoplasmy, which is of critical importance with regard to genetic stability. The T1 progeny plants of the transplastomic lines were shown to remain heteroplastomic, even after the continuous selection throughout the regeneration and vegetative/reproductive growth cycles on streptomycin-containing medium and soil. Our results indicate that the application of selection pressure after the transformed callus has been regenerated into a plantlet may not be an effective method for the achievement of homoplasmy. All of the homoplastomic

plants produced thus far had been selected under light conditions, through a repeated process of subculturing of transformed leaf tissues (Ruf *et al.*, 2001; Sidorov *et al.*, 1999; Sikdar *et al.*, 1998; Skarjinskaia *et al.*, 2003; Svab and Maliga, 1993) or transformed calli/cells (Dufourmantel *et al.*, 2004; Kumar *et al.*, 2004a; 2004b). However, this subculturing process can not be applied to cereal crops for prolonged periods under light conditions, as it results in the rapid reduction of regenerability in the transformed tissues. Therefore, further selection pressure must be applied at the undifferentiated callus stage until plastid segregation is complete to achieve homoplastomic status, as it was achieved in the carrot plastid transformation (Kumar *et al.*, 2004a).

Alternatively, in order to achieve homoplasmy, it may be necessary to develop new selection markers for a monocot-specific selection scheme. The use of suitable selection markers has been shown to be critical for success in the transformation of plastids in tobacco and cotton (Carrer *et al.*, 1993; Kumar *et al.*, 2004b; Svab and Maliga, 1993). Another strategy might be to use transgenic rice plants with a reduced number of plastids per cell as the transformation material, for the reduction of the selection period to achieve homoplasmy. In the *Arabidopsis* and tobacco plants, antisense suppression or over-expression of the *ftsZ* gene, which is involved in chloroplast division, has been shown to generate transgenic plants with fewer and larger chloroplasts in the mesophyll cells (Jeong *et al.*, 2002; Osteryoung *et al.*, 1998; Stokes *et al.*, 2000). However, it remains to be determined whether the same approach can generate transgenic rice plants with fewer chloroplasts per cell.

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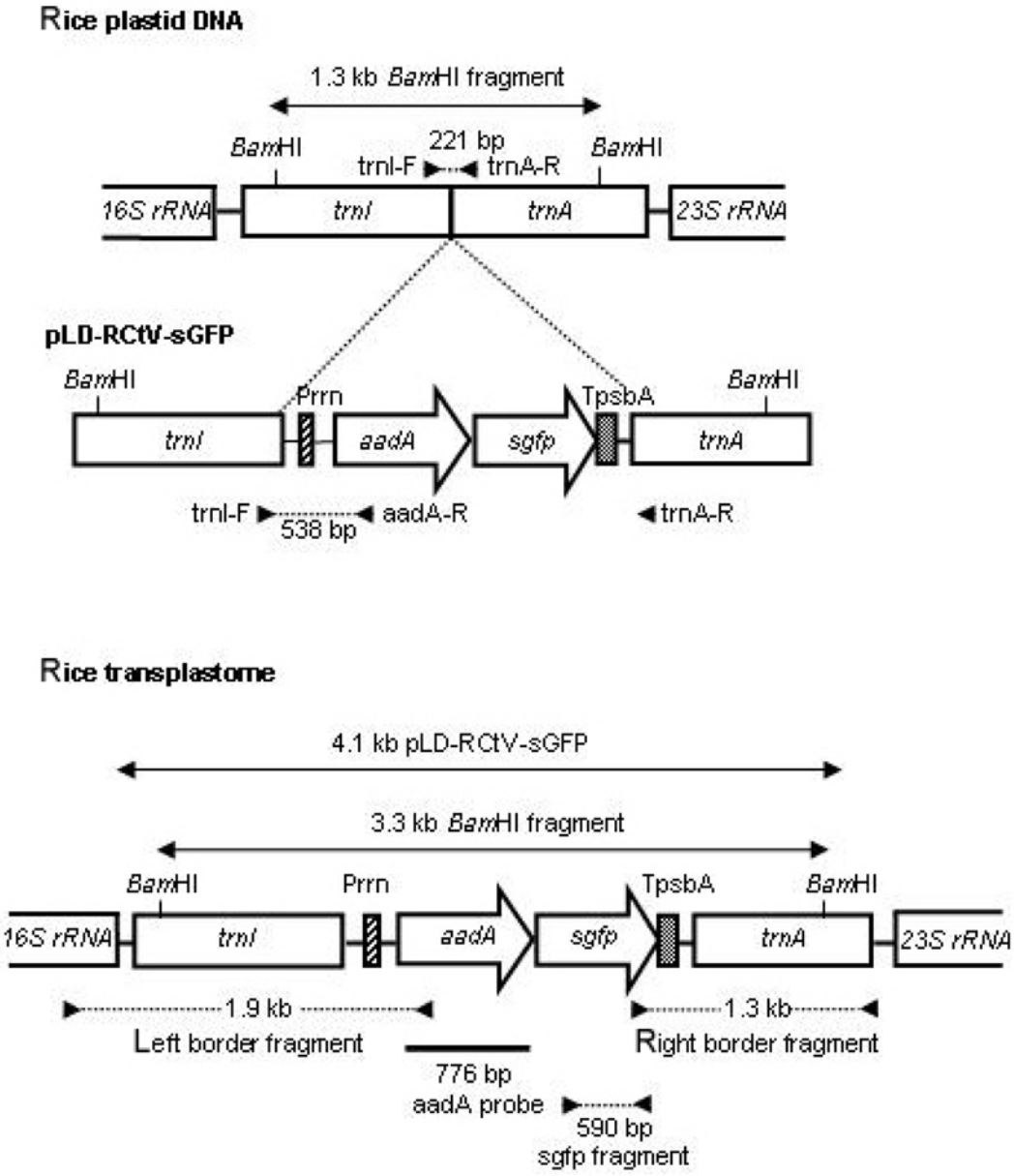

Fig. 1.

Rice transplastome resulting from the homologous recombination between the rice plastid transformation vector, pLD-RCtV-sGFP, and rice plastid DNA. The *aadA* and *sgfp* genes are driven by the rRNA operon promoter (Prrn). TpsbA indicates the 3′ UTR of the *psbA* gene. The filled triangles indicate the primer sites used for PCR analyses. The probe used for Southern analysis is marked with a thick line.

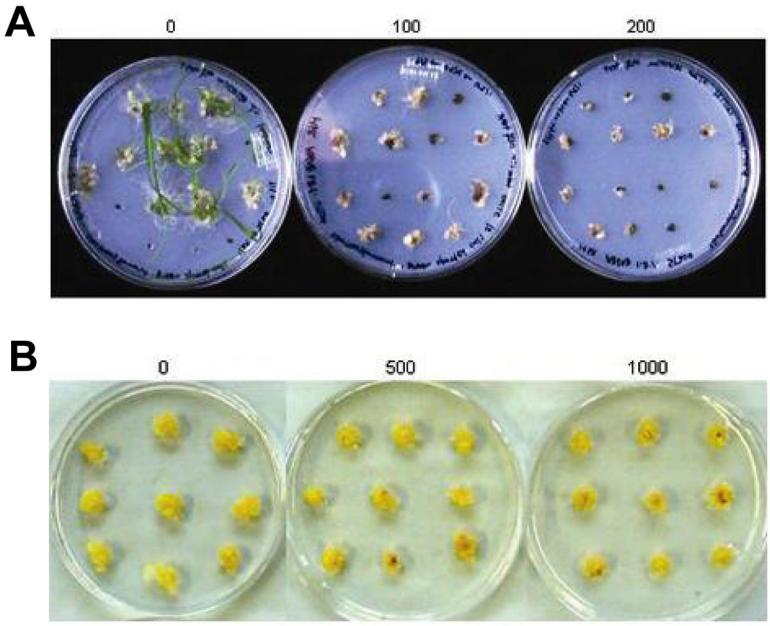

Fig. 2.

Determination of the optimal streptomycin concentration for inhibition of callus growth and shoot induction. The growth response of the total calli (2–3 weeks after germination) on selective regeneration medium containing (A) 0 (left), 100 (middle) and 200 (right) mg/L of streptomycin, after 2 months of culture in the light condition, and (B) 0 (left), 500 (middle) and 1,000 (right) mg/L of streptomycin, after 1 month of culture in the dark condition.

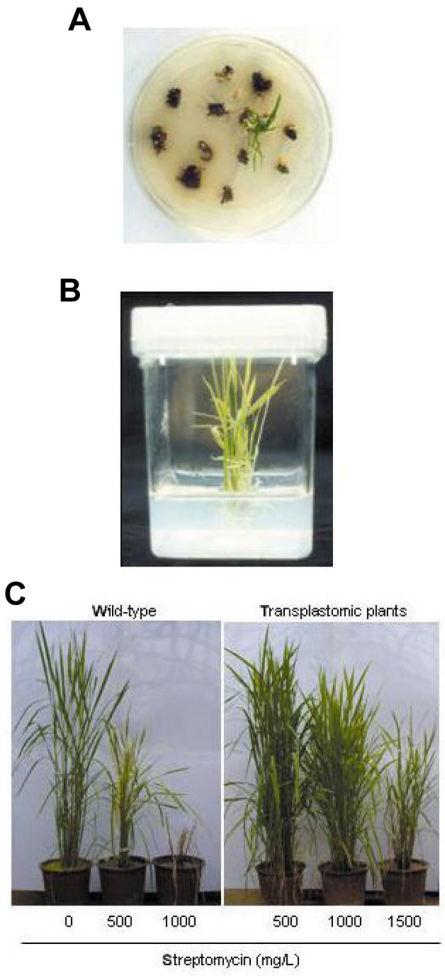

Fig. 3.

Recovery of transplastomic rice plants. (A) shoots induced from the bombarded calli after 2 months' incubation on regeneration medium containing 200 mg/L of streptomycin, (B) streptomycin-resistant shoots regenerated into a plantlet, approximately 1 month after transferring to rooting medium containing 500 mg/L of streptomycin and (C) phenotype of the mature transplastomic plants in the greenhouse, indicating streptomycin resistance at different concentrations.

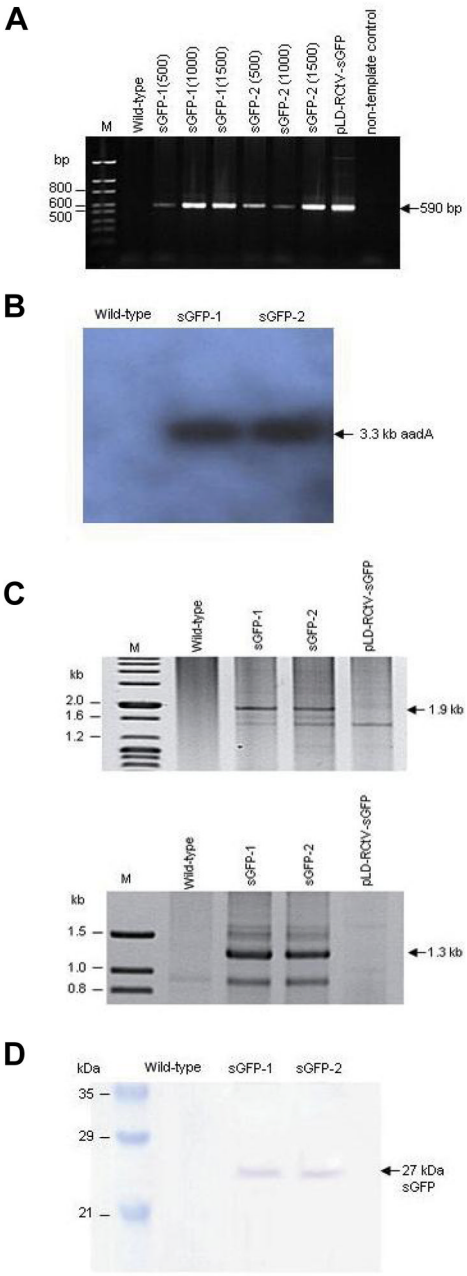

Fig. 4.

Characterization of T0 transplastomic plants. (A) PCR analysis indicates the presence of the 590 bp *sgfp* gene fragment in all six of the streptomycin-resistant plants derived from sGFP-1 and sGFP-2; streptomycin concentrations used in these selections are shown in parentheses, (B) Southern analysis using the *aadA* probe detects the 3.3 kb *Bam*HI fragment in both sGFP-1 and sGFP-2, (C) PCR amplification of 1.9 and 1.3 kb left and right border DNA fragments, respectively and (D) the detection of sGFP accumulation via Western analysis.

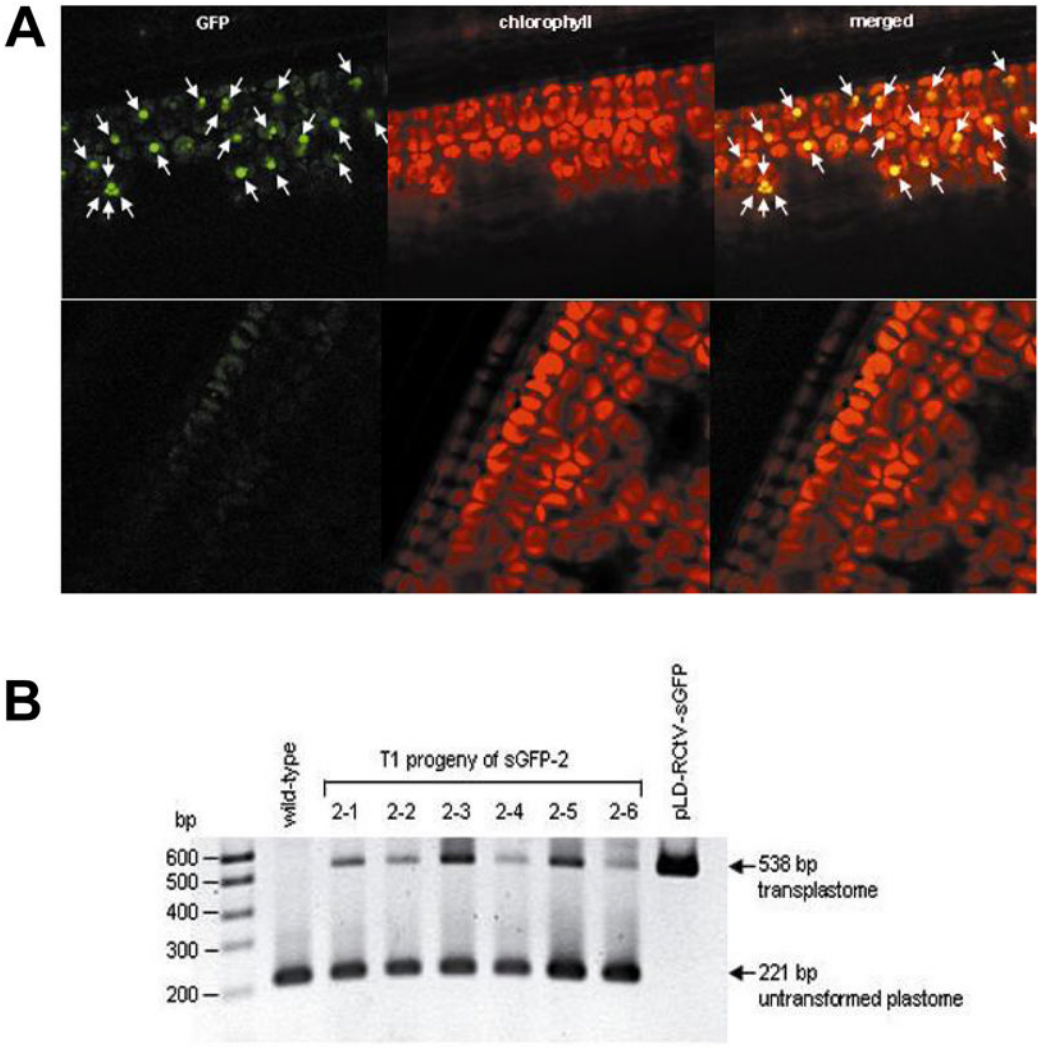

Fig. 5.

T1 progeny analyses. (A) Plastid-localized sGFP expression (marked with arrows) is observed from transplastomic line (top), whereas only a background level of autofluorescence is detectable from non-transformed plant (bottom) and (B) PCR analysis showing the presence of the 538 and 221 bp fragments, representing the transplastome and untransplastome, respectively.