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Generation of fertile transplastomic soybean

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Abstract

We describe here the development of a plastid transformation method for soybean, a leguminous plant of major agronomic interest. Chloroplasts from embryogenic tissue of *Glycine max* have been successfully transformed by bombardment. The transforming DNA carries a spectinomycin resistance gene (*aadA*) under the control of tobacco plastid regulatory expression elements, flanked by two adjacent soybean plastome sequences allowing its targeted insertion between the *trnV* gene and the *rps12/7* operon. All generated spectinomycin resistant plants were transplastomic and no remaining wild type plastome copies were detected. No spontaneous mutants were obtained. The transformation efficiency is similar to that of tobacco plastids. All transplastomic T0 plants were fertile and T1 progeny was uniformly spectinomycin resistant, showing the stability of the plastid transgene. This is the first report on the generation of fertile transplastomic soybean.

Introduction

Higher plants possess three different genomes each found in specific cellular compartments: the nucleus, the plastids and the mitochondria. Compared with classical, nuclear transformation, the integration of transgenes into the plastome offers many advantages, highly reviewed (Bock and Hagemann, 2000; Bogorad, 2000; Heifetz, 2000; Maliga, 2002, 2003). Plastids are present in all cells and their differentiation is determined by the function of the tissue: undifferentiated proplastids in immature meristematic cells, chloroplasts specialised in photosynthesis in green tissues, plastids involved in synthesis and storage of different metabolites such as starch in the amyloplasts, fatty acids in elaioplasts or carotenoids in chromoplasts. Plastids derive directly or indirectly from the differentiation of the same organelle, the proplastid, and therefore carry the same genetic information. The plastid genome (plastome) is a circular double stranded DNA molecule, in most higher plants between 120 and 170 kb, generally made up of two inverted repeated regions separating two single copy regions, harbouring about 130 genes. In higher plants, photosynthetic cells contain up to 100 chloroplasts, which in turn carry as many as 100 identical copies of the plastome (Bendich, 1987). This high level in plastome ploidy, in combination with the use of appropriate regulatory sequences, often results in high levels of transgene expression, so that the corresponding protein can account for up to 45% of the total soluble cellular proteins (De Cosa *et al.*, 2001). Many plastid genes are organised in operons, certainly because of the prokaryotic origin of this organelle, permitting the expression of several transgenes from one transcription unit (Shinozaki *et al.*, 1986). Further-

more, transgene integration in the plastid genome occurs by a double homologous recombination, thus avoiding position effects. No silencing has been reported yet in the plastid genome. Finally, for most higher plants, the plastid inheritance is maternal, reducing transgene scattering by pollen (Polans *et al*., 1990; Daniell *et al*., 1998). Nevertheless, this plastome inheritance needs to be analysed for each species since paternal or biparental transmissions have also been reported (Mogensen, 1996).

Although plastid transformation is very attractive, this technology is routinely used only in tobacco (Svab and Maliga, 1993). Recently, successful plastid transformation was reported in *Lycopersicon esculentum* (Ruf *et al*., 2001) and in *Lesquerella fendleri* (Skarjinskaia *et al*., 2003) which are the only other transplastomic plants that have been shown to be fertile and capable of transgene transmission to the next generation. Plastid transformation of *Arabidopsis thaliana* (Sikdar *et al*., 1998) has also been achieved but these plants were sterile. No data on the fertility of transplastomic *Solanum tuberosum* (Sidorov *et al*., 1999), *Oryza sativa* (Khan and Maliga, 1999), and oilseed rape (Hou *et al.,* 2002) is reported yet. Plastid transformation of soybean was also attempted, by Zhang *et al*. (2001a), but no whole and fertile plants were regenerated. It is important to emphasise that plastid transformation attempted in soybean and rice used non-leafy tissues, unlike the others transformed species. Difficulties to develop the plastid transformation technology for other major crops could be related to the limitations in currently available tissue culture systems and regeneration protocols. A main barrier in accomplishing this goal might also be the low level of plastid gene expression in non green totipotent cells such as those propagated *in vitro*.

Stable genetic transformation of plastids is obtained through three main steps: the first one is the introduction of the transforming DNA by a physical process: PEG treatment (O'Neill *et al*., 1993; Golds *et al*.,1993), biolistics (Svab *et al*., 1990) or microinjection (Knoblauch *et al*., 1999). This last method has not yet yielded stable transplastomic plants. During the second step, DNA integration occurs through a double homologous recombination (Boynton *et al*., 1988). The third step is an efficient selection of transplastomes by maintaining a strong selection pressure, homoplasmy being reached by sorting of the wild type (WT) and transformed genome copies. The most commonly used selectable marker is the *aadA* gene, which confers spectinomycin and streptomycin resistance (Svab and Maliga, 1993).

We report here the successful development of the first plastid transformation/selection/regeneration technology for the generation of fertile transplastomic plants of soybean. This is also to our knowledge, the first case of fertile transplastomic plants being obtained from non-leafy source tissue.

Materials and methods

Construction of the plastid transformation vector pCLT312

The soybean plastid transformation vector pCLT312 carries a heterologous expression cassette AADA-312 flanked by two *Glycine max* plastid DNA (ptDNA) fragments for the targeted integration, left homologous recombination region (LHRR) and right homologous recombination region (RHRR). The LHRR, containing the intergenic region of soybean plastome, between *trnV* and *rps12/7* genes (nucleotides 1922–3068; GenBank accession number X07675), was amplified by PCR from total DNA of *Glycine max*, cv ''Jack'', using two specific primers, OSSG5 (5′-ctagtggtaccgatccaa tcacgatcttctaataagaac-3′) and OSSG3 (5′-ctctc catgggttaacaagcttaactgctctatcggaaataggattgacc-3′). The RHRR, containing *trnV* and the 5′ end of the *16S rDNA* genes, (nucleotides 173 – 1921; X07675), was amplified by PCR from total DNA of *Glycine max* using two other specific primers: OSSD5 (5′-g tcgaccatggactagtccaccgcggtggtctagactcgaggacaatg gaatccaatttttcc-3′) and OSSD3 (5′-ctaggagctc caccgccgtatggctgaccg-3′). RHRR and LHRR were cloned in a pPCR-Script Amp SK(+) Vector (Stratagene), to form, respectively, pCLT308 and pCLT309. The *Kpn*I–*Kpn*I LHRR fragment was cloned into the *Kpn*I site of pCLT308, upstream of the RHRR, forming the pCLT300. An expression cassette was then cloned as an *Hin*dIII-*Xho*I fragment between the two plastid regions into the pCLT300, digested with *Xho*I and *Hin*dIII, to yield the pCLT311 vector. This expression cassette contained the coding sequence of a gene of interest transcribed from a synthetic promoter consisting of

the promoter of the tobacco *rRNA* operon (*Prrn*) fused with the 5′ untranslated region of the tobacco plastid *rbcL* gene, as described by Svab and Maliga (1993). The 3′ end of the *Prrn* contained a *Nco*I restriction site. The 3′*psbA* regulatory region (with a *Xba*I restriction site at the 5′ end) was used to stabilise the mRNA of the gene of interest (Svab and Maliga, 1993). The coding sequence of the *aadA* gene (a gift of Dr. Michel Goldschmidt-Clermont, University of Geneva, Switzerland; Goldschmidt-Clermont, 1991), containing a *Nco*I restriction site at the ATG start codon and a *Xba*I site following the stop codon, was cloned into the pCLT311 by digestion with *Nco*I and *Xba*I to generate the AADA-312 expression cassette and the transforming vector pCLT312 (Genbank accession number AY575999) (Figure 2).

Plant material

Embryogenic tissues from *Glycine max* L. Merr., cv "Jack" were initiated as described by Santarém and Finer (1999). Following the first induction on "D20", embryogenic tissues were transferred to FNL medium, derived from Samoylov *et al.* (1998) which had the following composition (mg/l): Na_2EDTA: 37.24; $FeSO_4$, $7H_2O$: 27.84; $MgSO_4$, $7H_2O$: 370; $MnSO_4$, H_2O: 16.9: $ZnSO_4$, H_2O: 8.6; $CuSO_4$, $7H_2O$: 0.025: $CaCl_2$, $2H_2O$: 440; KI: 0.83; $CoCl_2$, $6H_2O$: 0.025; KH_2PO_4: 170; H_3BO_3: 6.2; Na_2MoO_4, $2H_2O$: 0.25; myo-inositol: 100: nicotinic acid: 1; pyridoxine–HCl: 1; thiamine–HCl: 10; $(NH_4)_2SO_4$: 460; KNO_3: 2820; asparagine: 670; 1% saccharose; 2,4-D: 10; 0,3% gelrite; pH 5.7.

This medium enabled us to more rapidly cultivate tissues for bombarding, produced a higher embryogenicity and shortened transfer cycles. Green and compact embryogenic calli were then selected after 3–4 cycles on FNL medium and used for transformation.

Transformation, selection and regeneration of transplastomic soybean plants

Tissues were bombarded as described by Santarém and Finer (1999) using the PIG version (Particule Influx Generator) (Finer *et al.*, 1992). Basically 15–20 embryogenic calli were bombarded, on both sides, using 0.6 μm gold particles coated with 10 μg of pCLT312 DNA /bombardment, corresponding to two shots, precipitated with a mixture of 50 μl of 2.5 M $CaCl_2$ and 20 μl of 0.1 M spermidine free base as described by Russel *et al.* (1992). Two days after bombardment, calli were cut into very small pieces (about 1.5–2 mm diameter) and transferred to fresh FNL medium containing 200 mg/l of spectinomycin (or 300 mg/l of spectinomycin for the second experiment). Calli were transferred onto a fresh selection medium every fifteen days. First green spectinomycin resistant calli appeared after 8 weeks of selection. These putative transformants were amplified in a SBP6 liquid medium with 150 mg/l of spectinomycin (Finer and Nagasawa, 1988). Calli were converted into embryos using the medium described by Finer and McMullen (1991), containing 150 mg/l of spectinomycin. After 2 months on this medium, embryos were dessicated for 2 days and then transferred for germination to MS medium (Murashige and Skoog, 1962) at half ionic strength, containing 15 g/l of saccharose, 150 mg/l of spectinomycin and 7 g/l phytagar, pH 5.7. When young plants were well developed, they were transferred into soil for a 10–15 days acclimatisation period, before being transferred into the greenhouse for development and seed production.

To test the transgene transmission to the progeny, seeds from two different lines (2 and 7) were sown into a MS medium with half ionic strength containing 15 g/l saccharose and 500 mg/l of spectinomycin.

DNA extraction, PCR and southern blot analysis

Total DNA of calli or leaves was extracted with the Dneasy Plant Mini Kit (Qiagen). PCR analyses were performed in a Perkin-Elmer, GeneAmp PCR System 9600 Thermocycler with the Pow polymerase (Roche). Different sets of primers were used: OSSG3/OSSG5 and OSSD3/OSSD5 to amplify soybean plastid DNA, OSSG511 (5′-catgg gttctggcaatgcaatgtg-3′)/OSSG311 (5′-caggatcgaact ctccatgagattcc-3′) to detect WT plastomes in DNA isolated from transplastomic events. For Southern Blot analysis, the probes 1 and 2, hybridising, respectively, the left flanking homologous region and *aadA*, were obtained by PCR using respectively the following primers: OSSG5/OSSG310 (5′-gaac ctccttgcttctctcatgttacaatcctcttgccgc-3′) and OAAX3 (5′-ctcagtactcgagttatttgccgactaccttggtgatctcgcc-3′) /OAAN5 (5′-gaagcttccatggcagaagcggtgatcgccgaag-

3′). 35 PCR cycles were used for each amplification (with a denaturation step of 45 s at 94 °C, a hybridisation step of 45 s at 60 °C for 1 min, and an elongation step at 72 °C with time dependant on the size of the desired fragment), after a denaturation step at 94 °C for 5 min and finally, a 5 min elongation step at 72 °C finished the PCR amplification.

Southern Blot analyses (Sambrook *et al*., 1989) were performed with 1 μg of total DNA extracted from 10 transplastomic calli. DNA was digested by *Eco*RI (Biolabs). DNA fragments were separated by over night electrophoresis (55 V) in a 0.8% agarose gel (QA Agarose TM Multipurpose, QBIOGENE), before being transferred to nylon membrane (Hybond N+, AMERSHAM) under alkaline conditions, as described in Sambrook *et al*. (1989). DNA fragments were revealed by radioactive probes (^{32}P) hybridisation. These probes were amplified by PCR with primer pair OSSG5/OSSG310 for the one hybridising on intergenic region (on LHRR) and primer pair OAAX3/OAAN5 for the other hybridising on *aadA* coding sequence. They were purified using PCR Purification Kit (Qiagen) and labelled by random priming with a Megaprime kit (AMERSHAM). Membranes were washed with 6XSSC, 2XSSC 0.1% SDS and 0.1XSSC 1% SDS solutions, at 65 °C. Autoradiograms were obtained after 2 h exposure, at −80 °C, with an intensification screen.

Results

Optimisation of a selection protocol

For the development of an efficient protocol of soybean plastid transformation, we first established an efficient selection system. The most commonly used selection for high-frequency plastid transformation in higher plants uses a bacterial *aadA* gene encoding an aminoglycoside-3″-adenylyltransferase conferring spectinomycin and streptomycin resistance (Svab and Maliga, 1993; Sikdar *et al*., 1998; Khan and Maliga, 1999; Ruf *et al*., 2001; Skarjinskaia *et al*., 2003). Therefore, in order to determine the appropriate antibiotic (spectinomycin or streptomycin) and the suitable concentration for selection, soybean embryogenic tissues were transferred to FNL media containing various concentrations of spectinomycin and streptomycin. Soybean tissues appear to be naturally tolerant to streptomycin (up to 800 mg/l of streptomycin, data not shown) and sensitive to spectinomycin (Figure 1). Tissues start to bleach at 50 mg/l spectinomycin, fifteen days after selection. Complete bleaching is observed at 200 mg/l of spectinomycin. This concentration was used to select transformants. Zhang *et al*. (2001a) described a different process of selection of the transplastomic events, where the spectinomycin concentration was increased from 100 to 400 mg/l spectinomycin, during the 2 months of selection.

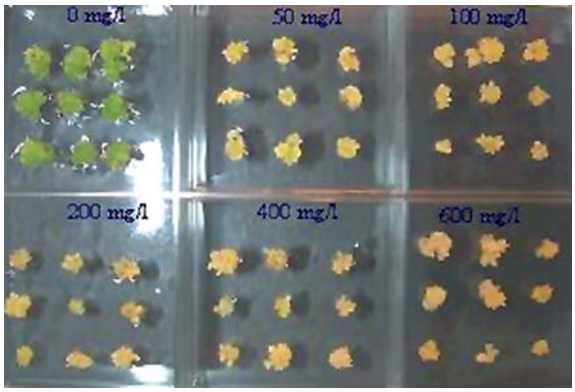

Figure 1. WT embryogenic calli of soybean on FNL medium with various spectinomycin concentrations, 1 month after treatment.

Construction of the pCLT312 plastid transformation vector

The transgene integration in the plastome occurs by double homologous recombination. Therefore, we first had to determine an appropriate integration site. In *N. tabacum* (Svab *et al*., 1990; Sikdar *et al*., 1998; Staub *et al*., 2000), *O. sativa* (Khan and Maliga, 1999), *S. tuberosum* (Sidorov *et al*., 1999), *A. thaliana* (Sikdar *et al*., 1998) and *Brassicaceae* (Hou *et al*., 2003; Skarjinskaia *et al*., 2003), the insertion sites of foreign genes used (reviewed in Maliga, 2004) included the *rbcL-accD* (Svab and Maliga, 1993; Daniell *et al*., 1998; Kota *et al*., 1999), the *trnV-rps12/7* (Sikdar *et al*., 1998; Staub *et al*., 2000 and other reports), the *trnI–trnA* (De Cosa *et al*., 2001), the *rps7-ndhB* (Hou *et al*., 2003) and *ndhF-trnL* (Zhang *et al*., 2001c) intergenic regions. The soybean plastome is not completely sequenced yet. Plastid DNA sequences

available in databases show an organisation different from the tobacco plastome but similar to that of *Lotus japonicus*, another leguminous plant. For instance, the *rbcL* and *accD* genes, adjacent in the tobacco plastome exhibit different locations on the soybean plastid DNA (*accD* is located between *psaI* and *rps16* genes). This observation makes the integration of a transgene between the *rbcL* and *accD* genes impossible without deleting a part of the plastome. Since the sequence of the intergenic region between the *trnV* and *rps12/7* genes in *Glycine max* plastome is known, plastid transformation vectors were designed to target the selection cassette in this inverted repeated region. Therefore, two *Glycine max* ptDNA fragments (RHRR and LHRR) containing respectively the 5′ end of the *16S rDNA* gene, *trnV* and the intergenic region upstream of the *rps12/7* operon were amplified by PCR (Figure 2).

After the sequencing of PCR amplified fragments, the sequence of LHRR appeared to be different from that which is published. The presence of mismatches and an additional sequence of 217 bp were observed (Genbank accession number AY575999). Comparison with other plastid genomes showed that this additional sequence is present in other species such as *Lotus japonicus* and *Arabidopsis thaliana*. Sequence variations in the non-coding region of soybean plastid DNA were previously described by Xu *et al.* (2000) in the genus *Glycine*. The plastid transformation vector, pCLT312, also contains a selection cassette. The selectable gene, *aadA*, is under the control of tobacco plastid regulatory elements: the 16S ribosomal RNA operon promoter (*Prrn*) fused to the 5′ UTR of the *rbcL* gene, and the 3′ end of *psbA* gene (described by Svab and Maliga, 1993) (Figure 2). This tobacco selection cassette has been successfully used for the plastid transformation of other species (Svab and Maliga, 1993; Sikdar *et al.*, 1998; Ruf *et al.*, 2001; Skarjinskaia *et al.*, 2003).

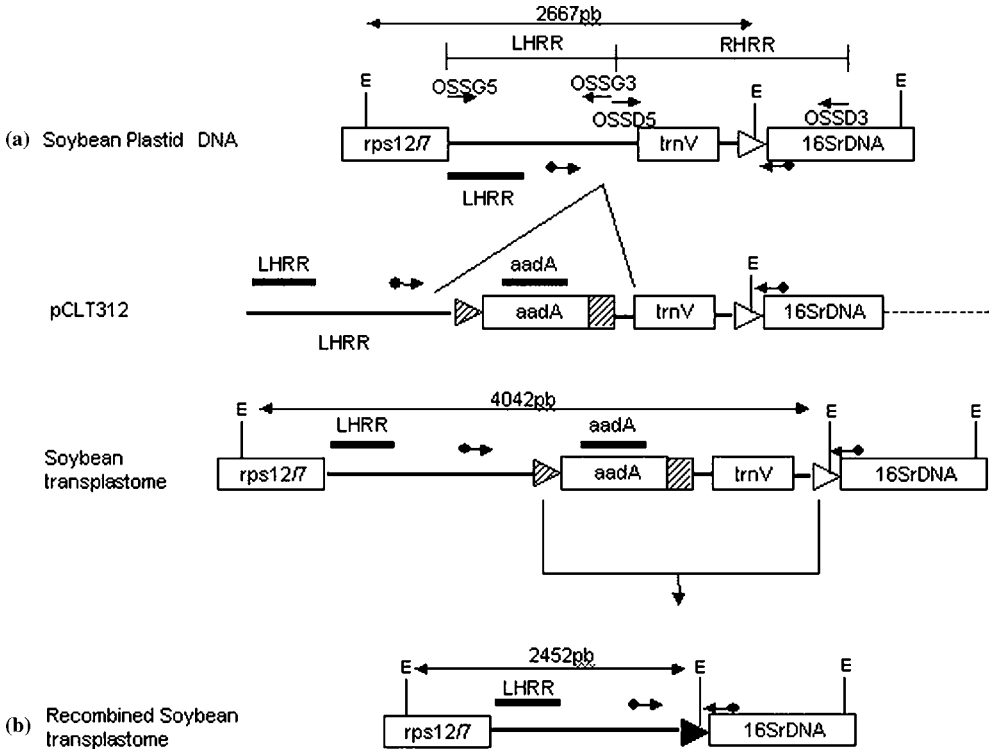

Figure 2. Integration of the *aadA* gene from vector pCLT312 into the wild type soybean plastome to yield transplastome (a) and recombined transplastome (b) ➞ Primers used to amplify the soybean LHRR and RHRR; ●➞ Primers used to detect WT and recombinant plastomes for PCR analysis (●➞ OSSG511) (➞● OSSG311); E *Eco*RI restriction sites; ▬ Probes used on Southern Blot; ▷ Soybean *Prrn*; ▷ *N. tabacum Prrn*; ▶ Recombined *Prrn* is the chimeric promoter resulting from homologous recombination between the tobacco and soybean *Prrn*; ▨ *N. tabacum* 3′UTR *psbA*.

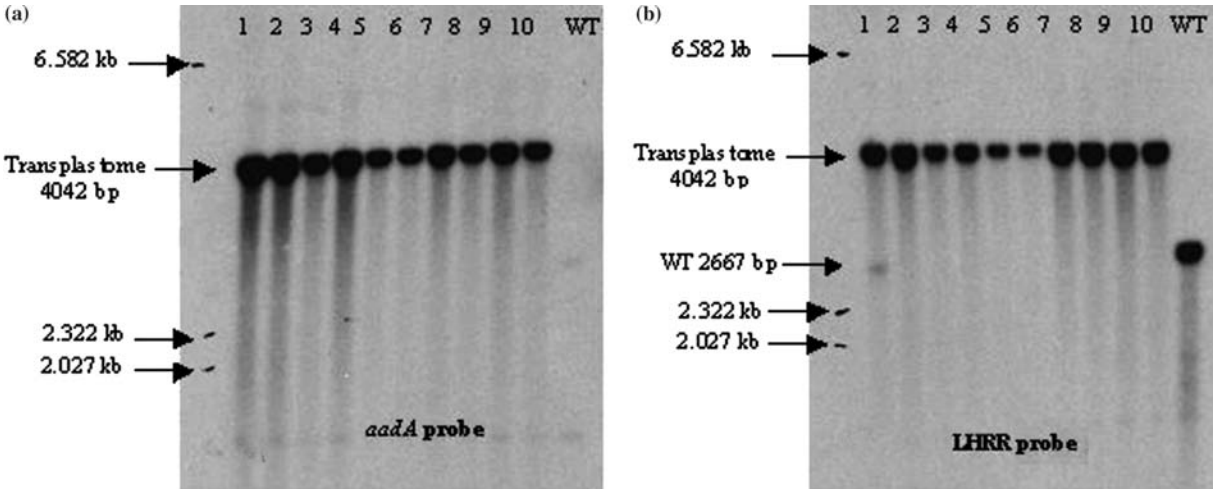

Figure 3. Southern Blot analysis. Total DNA extracted from transplastomic events (events 1–10, lanes 1–10) and WT soybean was digested by *Eco*RI. Blots were probed with the LHRR (LHRR, Figure 2) and the *aadA* coding region (*aadA*, Figure 2).

Generation and analysis of soybean transplastomic plants

Plastid transformation experiments were carried out by bombardment of soybean embryogenic tissue, using gold particles coated with pCLT312. Two days after bombardment, tissues were transferred onto a medium containing the selection agent (200 mg/l spectinomycin) and sub-cultured on the same medium every 2 weeks. The first spectinomycin-resistant calli appeared after a period of 2 months (Figure 5a).

Eleven spectinomycin-resistant calli were obtained from four bombardments. A preliminary PCR analysis showed that all of them were transplastomic, each containing the *aadA* gene properly integrated in the plastome (data not shown). No spontaneous mutants were detected among these spectinomycin resistant calli.

Southern blot analysis was performed to study the level of heteroplasmy in soybean transplastomic lines. Total cellular DNA extracted from ten transformed calli was digested with *Eco*RI, electrophoresed and subsequently blotted onto nylon membrane. The *Eco*RI-digested DNA was then hybridised with the LHRR sequence and the *aadA* probes (Figure 2). The *aadA* probe revealed a 4042 bp band for all transformed calli, indicating that all of them were transplastomic, and that the transgene was integrated at the expected site in the plastome (Figure 3). This was confirmed by the patterns obtained with the LHRR probe. In addition, the 2667 bp band corresponding to the wild type plastome, was detectable in the WT line and only for the callus 1, for which the spectinomycin selection was omitted for 10 days, 61 days after bombardment, suggesting that all the others were homoplasmic, or at least very close to homoplasmy (Figure 3).

The transformation procedure was repeated a second time using the same conditions, except that the spectinomycin concentration was raised to 300 mg/l after one month of selection. Five spectinomycin resistant calli were obtained and analysed by PCR, with the primer pair OSSG511/OSSG311 (Figures 2 and 4). These PCR primers hybridise to sequences flanking the transgene insertion site of *Glycine max* plastome. This DNA amplification can give two PCR products of 2406 and 1031 bp for the transplastome and the WT plastome, respectively (Figures 2 and 4). All the new spectinomycin resistant calli were transplastomic. Again no spontaneous mutant was observed. Moreover, no amplification of a wild type specific fragment was obtained from the total DNA extracted from callus 14, unlike all other calli. This result suggests that the event 14 could already be homoplasmic at the callus state. Plants were regenerated, *via* somatic embryogenesis, from all five calli (Figure 5b).

The spectinomycin concentration for the transplastomic callus 12 of this second bombardment experiment was further increased, to test if the selection pressure could influence the

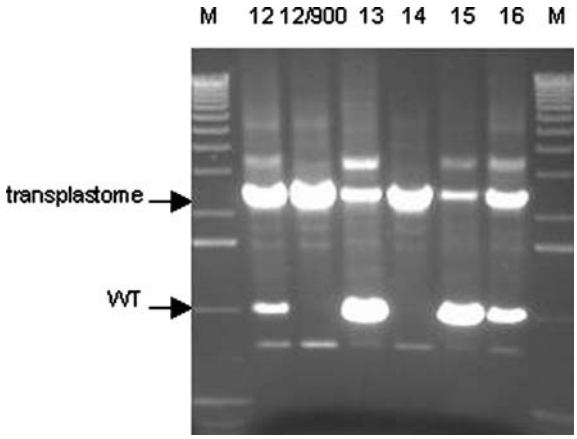

Figure 4. PCR analysis with the primer pair OSSG511/OSSG311 on total DNA of each transplastomic callus (lanes 12–16: events 12–16). The event 12 was selected on FNL media containing either 300 mg/l (lane 12) or 900 mg/l (lane 12/900) of spectinomycin.

heteroplasmic state. PCR analysis performed with total DNA isolated from callus 12 (primers OSSG511/OSSG311), grown with different spectinomycin concentrations (300 and 900 mg/l), showed that a strong selection pressure could reduce the amount of wild type plastome copies (Figures 2 and 4).

Study of transgene stability

Plants were regenerated and grown to maturity in the greenhouse. All transplastomic plants were fertile and produced seeds. In order to check the *aadA* stability, the T1 progeny of the transplastomic plants 2 and 7 was tested for its ability to grow on spectinomycin. One hundred seeds of both these plants were sown *in vitro*, on a medium containing 500 mg/l spectinomycin. As expected for a plastid transgene, the T1 progeny was uniformly spectinomycin-resistant (Figure 5c). T1 seeds of different transplastomic events (1, 2, 3, 7, 8 and 9) were sown in soil without selection pressure, and grown in the greenhouse for further characterisation.

PCR analysis was performed to detect the few remaining wild type plastomes that may still be present and undetectable by Southern blot because of the detection limit. A PCR amplification was performed on total DNA from calli, T0 and T1 leaves from the events 1 to 10 using specific primers OSSG511/OSSG311 (Figures 2 and 6). The 2406 bp amplification product was obtained in each case, confirming the integration of the transgene in the plastome of each spectinomycin resistant event, and the absence of spontaneous mutants. A small product, specific to WT plastome, was strongly amplified with the total DNA of callus 1. This result is consistent with the Southern Blot analysis as this transplastomic callus was the only one in which wild type copies could be detected.

No PCR fragment specific to the WT plastome was amplified subsequently in the regenerated plant from the callus 1, indicating that the

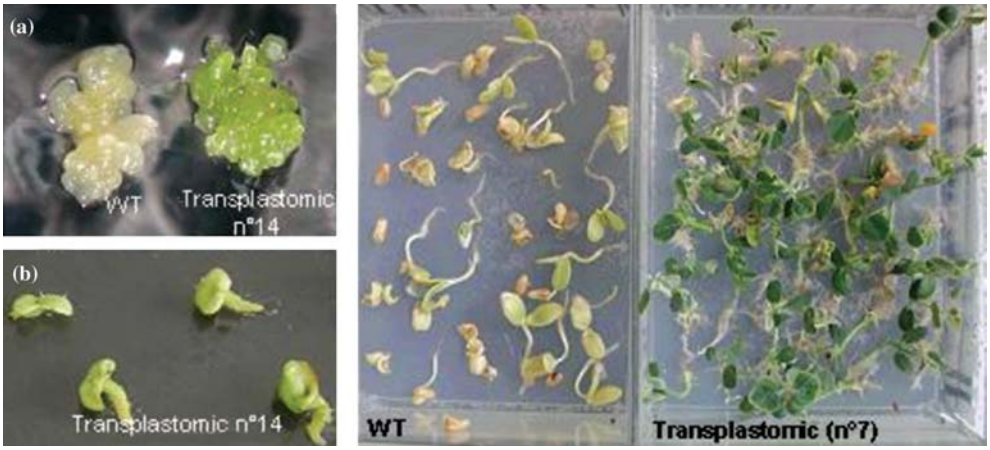

Figure 5. (a) Primary transformants, after 2 months on FNL + 200 mg/l of spectinomycin; (b) somatic embryos after 30 days on conversion medium (150 mg/l of spectinomycin); (c) *in vitro* germination of WT and transplastomic seeds on medium containing 500 mg/l of spectinomycin, after 20 days.

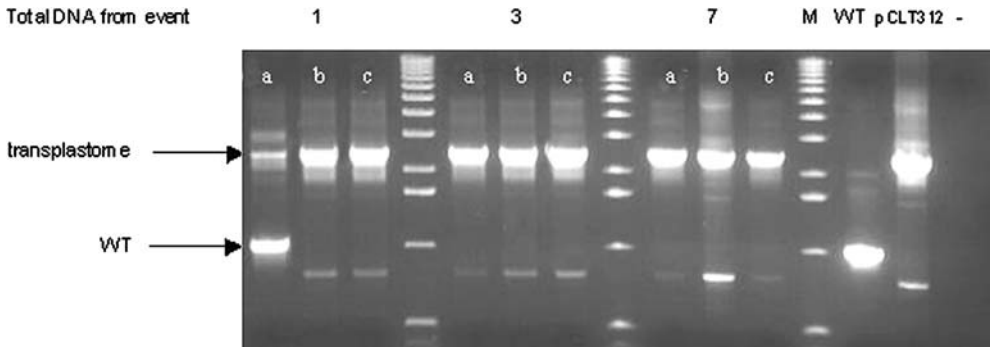

Figure 6. PCR analysis with the primers OSSG511/OSSG311 on the transforming vector (pCLT312), on total DNA extracted from WT soybean leaves and on total DNA extracted from transplastomic callus (lane a), T0 and T1 leaves (lanes b and c, respectively) of the events 1, 3 and 7.

number of WT plastomes had severely decreased during the regeneration process of this transplastomic event. We could not detect any difference between the generations T0 and T1, exemplifying the stability of the transgene through the first generation.

Finally, a smaller fragment than the WT amplicon was also observed. Its size is consistent with a homologous recombination event of the transplastome between the soybean *Prrn* promoter driving the endogenous *16S rDNA* gene and the tobacco *Prrn* driving the *aadA* transgene, resulting in a recombined transplastome (Figure 2b). A similar homologous recombination event between two direct *Prrn,* leading to the exogenous *aadA* and endogenous *trnV* deletions from the plastid genome, has also been described by Hajdukiewicz *et al*. (2001) and Corneille *et al*. (2001). However, in our case, this smaller DNA fragment was likely to be a PCR artifact since it was not detected in our Southern Blot analysis, and as it was also obtained by PCR analysis with pCLT312 transforming vector, extracted from a *recA*$^{-}$ *E. coli* strain (Figures 2 and 3).

Discussion

This report is, to our knowledge, the first description of a method allowing the efficient generation of stable and fertile transplastomic soybean plants. The plastid transformation of soybean was previously attempted without success by Zhang *et al*. (2001a). In our plastid transformation system, the transgene was targeted between the *trnv* gene and the *rps12/7* operon of *Glycine max* plastome. This is also, the first plastid transformation of non-leafy tissue, regenerated in fertile transplastomic plant. Transplastomic regenerated lines were fertile and homoplasmic. The progeny of these plants was uniformly spectinomycin-resistant, showing that the transgene integration is stable.

In the first transformation experiment, eleven transplastomic events were obtained from four shots (about 15–20 embryogenic calli per shot). In the second one, five transplastomic lines were obtained from four shots. The transformation efficiency achieved is thus around two transformants per shot, which is similar to the efficiency reported on tobacco (Svab and Maliga, 1993), and 15–100 fold higher than for potato (Sidorov *et al*., 1999), tomato (Ruf *et al*., 2001), *Lesquerella fendleri* (Skarjinskaia *et al*., 2003) and *A. thaliana* (Sikdar *et al*., 1998). Interestingly, for all these species, the use of spectinomycin selection for plastid transformants results in the occurrence of spontaneous mutants arising from specific point mutations in the plastid *16S rDNA* gene (Svab and Maliga, 1991). The proportion of spontaneous mutants (including nuclear integrations which could permit the transgene expression) varies from 20% for tobacco to 99% for *Lesquerella fendleri* (Skarjinskaia *et al*., 2003). In contrast, no spontaneous spectinomycin mutant appeared in our two independent experiments. The likely explanation could be that the point mutations on the *16S rDNA* do occur at a similar frequency than in other species, but, due to the context and/or a conformation of the *Glycine max* mutated 16S rRNA, these mutations do not result in a sufficiently high spectinomycin tolerance.

After 2 months of selection, homoplasmic recombinant calli were obtained. WT plastome copies were detected by Southern blot only in callus 1 for which the selection was omitted during 10 days. With our method of plastid transformation of soybean, transplastomic plants were obtained which were rapidly homoplasmic at the callus stage. This could be linked to the nature of the tissue we transformed: indeed, embryogenic tissues contain fewer and smaller plastids with lower amounts of plastid DNA than leaf tissues (Zhang *et al*., 2001b). Nevertheless, it remains difficult to estimate all factors involved in this rapid establishment of a homoplasmic state. All plastid transformations described up to now did not always result in homoplasmic plants at T0 generation. Some of them required several rounds of selection/regeneration, whilst others gave heteroplasmic plants (Khan and Maliga, 1999; Ruf *et al*., 2001; Skarjinskaia *et al*., 2003). These plastid transformation methods differed in many factors that could influence the establishment of a homoplasmic state, e.g. the species transformed, targeting sites in the plastome, selection cassettes (with different selection genes controlled by various expression regulatory elements), transformation process, selection pressure and the step of differentiation of the transformed tissue.

PCR analysis of transplastomic callus and T0 leaves of the event 1, showed that the copy number of WT plastome still present in this event decreased between these two stages of development. This decrease in WT plastome ratio during the regeneration procedure could be linked to the embryogenic step. During the somatic embryogenesis the plastid copy number could diminish, which could explain the diminution of WT plastome copies. Moreover, a weaker activity of the plastid encoded polymerase promoter in the somatic embryo could have participated to the increase of transplastome copies. Indeed, the PCR analysis carried out on total DNA extracted from calli, T0 and T1 leaves of the other events, and from T0 toT1 leaves of the event 1 did not reveal the presence of WT copies of plastid DNA.

The strength of selection pressure was tested on two separate transformants. For the first one, the selection pressure was removed for 10 days and gave a transplastomic callus containing much more WT plastome than the others which were always grown on a selection medium. The second transplastomic callus was selected and amplified on medium containing different concentrations of selection agent. The same callus grown with 900 mg/l of spectinomycin contained less WT genome than when grown on 300 mg/l selection agent. Thus, the influence of the selection pressure seems to be important to obtain homoplasmy. We also noticed that the increase of selection pressure between the two independent experiments seemed to be insufficient (200–300 mg/l) to influence the level of remaining WT plastome (contrary to the increase from 300 to 900 mg/l tested for one transplastomic callus in the second experiment). Further experiments should be performed to better characterise the influence of the strength of selection pressure on the homoplasmy establishment.

This organelle transformation method is clearly an alternative approach to improving crop performance or using plants beyond the farm, although the technology today is only available for a few species. The maternal inheritance of the plastid transgene for soybean has to be analysed. Pollination experiments are in progress to study the transgene transmission by pollen. Another aspect we will adress in the future concerns the pattern of expression of a transgene integrated in the soybean plastome, in different tissues and its level of expression. This could be performed on the existing material with antibodies directed against the selectable marker AADA, or on new material currently being generated with genes of interest.The plastid transformation of soybean can be used to introduce agronomic traits like insecticide resistance (Kota *et al*., 1999) or herbicide tolerance (Ye *et al*., 2001). This technology can also be used for metabolic engineering or for molecular farming (Daniell *et al*., 1994; Staub *et al*., 2000). Because of these potential applications in a crop of agronomic interest such as soybean, the use of a non-antibiotic selectable marker (Daniell *et al*., 2001) could be developed. Another alternative could be the development of a technology of elimination by homologous recombination between two direct repeated sequences (Iamtham and Day, 2000) (recombination system that could be forced by a negative selection (Serino and Maliga, 1997)) or with a site specific recombination system such as Cre–Lox (Corneille *et al*., 2001; Hajdukiewicz *et al*., 2001).

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