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# Plastid Transformation of Soybean Suspension Cultures

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## Abstract

**Plastid transformation was attempted with soybean [*Glycine max* (L.) Merr.] leaves and photoautotrophic and embryogenic cultures by particle bombardment using the transforming vector pZVII that carries the coding sequences for both subunits of *Chlamydomonas reinhardtii* Rubisco and a spectinomycin resistance gene (aadA). Spectinomycin resistant calli were selected from the bombarded leaves but the transgene was not present, indicating that the resistance was due to mutations. The *Chlamydomonas* rbcL and rbcS genes were shown to be site-specifically integrated into the plastid genome of the embryogenic cells with a very low transformation efficiency. None of the transformed embryogenic lines survived the plant regeneration process so no whole plants were recovered. This result does indicate that it should be possible to insert genes into the plastid genome of the important crop soybean if the overall methods are improved.**

## Introduction

Nuclear transformation of plants has already revolutionized plant biology research and will continue to have an increasingly significant impact. However, making transgenic plants by transforming the plastid genome (Maliga et al., 1993) provides not only another avenue of investigation into nuclear-organelle interaction, regulation of photosynthesis-related gene expression, and molecular evolution, but is also a potentially effective tool for improving crop traits such as photosynthetic properties and nutritional value. Plastid transformation may also be important for mass-production of valuable products from plant metabolic processes. Since the plastid genome is maternally inherited in most crop species, transgenes in the plastid genome will usually not spread to other sexually compatible species.

Tissue culture is an essential step of transformation for most plants. Due to their ability to regenerate whole plants, cultured soybean embryogenic tissues, first developed by Finer and Nagasawa (1988), have become one of the two most commonly used materials for nuclear transformation in soybean (Finer and McMullen, 1991; Stewart et al., 1996). The soybean photoautotrophic (PA) cell line (SB-1) was established by Horn et al. (1983). It has since become a convenient and important system for physiological, biochemical and molecular biology studies (summarized in Widholm, 1992 and other later studies such as Terzaghi et al., 1997 and Heo et al., 1999), even though the PA cells cannot be regenerated into plants.

An important function of the chloroplast is photosynthesis, a process directed and regulated by the expression and interaction of both nuclear- and plastid-encoded genes. The key enzyme for $CO_2$ fixation is ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The large subunit of Rubisco (rbcL) is encoded by the plastid genome, whereas the small subunit (rbcS) is encoded by nuclear genes. Our goal is to explore a new way of increasing the photosynthetic potential, i.e. the capacity of $CO_2$ fixation, in soybeans by genetically modifying

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Rubisco through chloroplast transformation. We attempted to replace the soybean plastid rbcL gene with the genes for the green alga *Chlamydomonas reinhardtii* Rubisco that shows a higher net photosynthetic rate under high $CO_2$ conditions. We hoped to generate transplastomic soybean plants that may perform more efficient photosynthesis in the high atmospheric $CO_2$ environment projected for the near future. Embryogenic tissue, PA cells and leaves were used as targets for particle bombardment.

## Materials and Methods

### Plant materials and cell-suspension cultures

Tissue cultures of soybean [*Glycine max* (L.) Merr.] were maintained as described by Zhang et al. (2001).

### Construction of soybean plastid transformation vector

A coding sequence of *Chlamydomonas reinhardtii* Rubisco large subunit was fused with the promoter region of the soybean rbcL gene so that the *Chlamydomonas* rbcL gene has an identical promoter sequence as the wild type soybean rbcL gene. The selectable marker gene aadA (conferring spectinomycin resistance, kindly provided by J. Boynton) was driven by the strong constitutive plastid 16S rRNA operon promoter (Prrn, 337 bp) from tobacco. The *Chlamydomonas* rbcS coding sequence without the transit peptide was fused to a 377-bp soybean rbcL promoter to achieve a comparable level of gene expression between the rbcL and rbcS of *Chlamydomonas*. The transgene cassette, including *Chlamydomonas* rbcL, aadA and *Chlamydomonas* rbcS, was ligated to the noncoding region of the soybean rbcL gene as termination region. Overall, the soybean plastid expression vector, pZVII, was flanked by soybean plastid atpβ-rbcL5'-noncoding region at one end and rbcL3'-noncoding region at the other end as anchoring sequences (Figure 1A). All the components in the vector were confirmed by DNA sequencing. During plastid transformation, the transgene cassette is expected to be inserted into the region between the soybean plastid atpβ/ε and trnL genes by homologous recombination. As a result, the soybean rbcL gene would be replaced by *Chlamydomonas* rbcL, and in addition, the *Chlamydomonas* rbcS gene and the aadA gene would be inserted into the soybean plastid genome (Figure 1A).

### Transformation and selection

The embryogenic tissue (cv. Jack) subcultured for 4-5 d

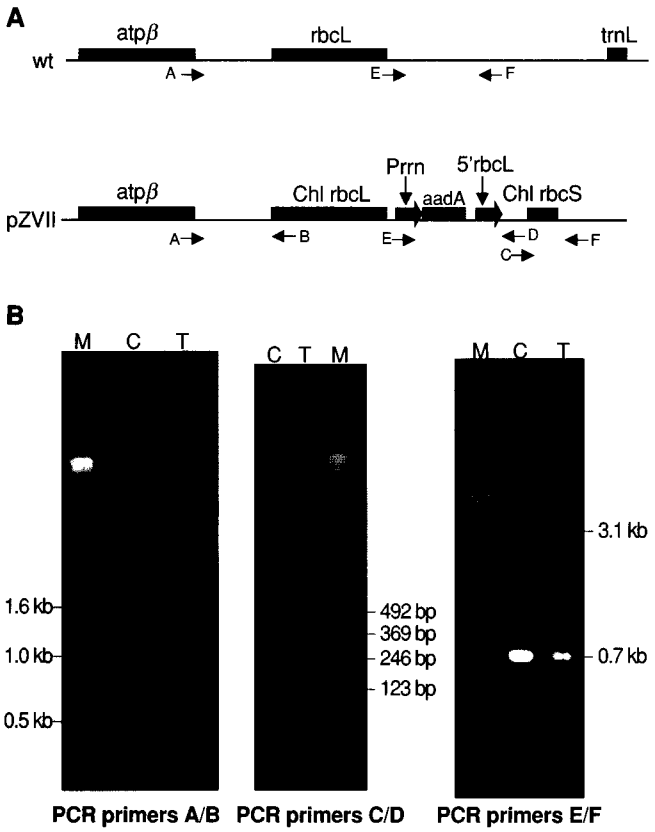


**Figure 1.** Transformation of the soybean chloroplast genome with *Chlamydomonas* Rubisco large and small subunit and aadA genes. A: Structure of the transformation vector pZVII and the cognate region of the plastid genome of wild type soybean. The promoter for the *Chlamydomonas* rbcL gene is identical to that for the wild type soybean rbcL. The promoters for aadA and *Chlamydomonas* rbcS genes are indicated. The location and direction of PCR primers is indicated. The figure size is not to scale, B: PCR confirmation of site-specific integration of the *Chlamydomonas* rbcL and rbcS into the chloroplast genome of the resistant soybean embryo. PCR with primers A/B amplified a 1.2-kb fragment flanking the soybean atpβ and *Chlamydomonas* rbcL genes. PCR with primers C/D identified a 0.4-kb portion of the *Chlamydomonas* rbcS coding sequence. The amplified DNA products were present only in the transformed embryo (T) and not in the untransformed control (C). PCR with primers E/F identified a 0.7-kb fragment in the wild type soybean cells (C) and a 3.1-kb fragment in the transformed embryo (T). The presence of the 0.7-kb fragment in the transformed tissue indicates that it was heteroplastomic. M: DNA markers.

was placed on Whatman filter paper and allowed to dry for at least 30 min in a covered but not sealed Petri dish in the laminar flow hood. Gold particles (average diameter: 0.6 μm; Bio-Rad) were coated with the transformation vector DNA (Svab et al., 1993). The bombardment was carried out with a PDS 1000/He Particle Gun (Bio-Rad) using 1350

psi rupture discs, under 27 to 28 inches of Hg vacuum. After bombardment, the embryogenic tissue was transferred into FG liquid medium (Finer and Nagasawa, 1988) and incubated at 27 to 28°C without shaking for 2 d. Then the flasks were placed on a shaker (130 rpm) for an additional 3 to 5 d. Afterwards, the embryogenic tissue was subcultured every 7 d in FG containing spectinomycin dihydrochloride sequentially at 100 μg/mL for 2 weeks, 200 μg/mL for 2 weeks and 300 μg/mL for 4 weeks. The cultures were maintained on 400 μg/mL of spectinomycin for up to 2 months. As a control, a portion of the bombarded embryogenic tissue was cultured as above, except in a medium without spectinomycin.

The PA cell SB-1 suspension culture (cv. Corsoy) and leaves (cv. Jack) were also used for bombardment. The PA cells collected on Whatman filters without the drying step were bombarded as described above and then returned to liquid $KN^0$ medium for 7 d. The cells were grown in medium with 100 μg/mL spectinomycin for one month, and then the spectinomycin was increased to 200 μg/mL. The PA cells were subcultured every two weeks. Young leaves of greenhouse-grown soybean plants (cv. Jack) were surfaced sterilized and placed abaxial side up on agar-solidified L2NK medium modified from that described by Phillips and Collins (1981). The L2NK contains 12.5 mM $NH_4NO_3$, 20.8 mM $KNO_3$, 2.4 mM $KH_2PO_4$, 4.5 μM KI, 0.6 mM $NaH_2PO_4$, 1.8 mM $MgSO_4$, 60 μM $MnSO_4$, 48.5 μM $H_3BO_3$, 7 μM $ZnSO_4$, 1 μM $NaMoO_4$, 1 μM $CuSO_4$, 1 μM $CoCl_2$, 4.1 mM $CaCl_2$, 100 μM NaFeEDTA, 8.1 μM nicotinic acid, 30 μM thiamine HCl, 4.9 μM pyridoxine HCl, 25 μM naphthaleneacetic acid, 10 μM kinetin, 1.4 mM myo-inositol, pH 5.8, and 73 mM (2.5%) sucrose. The bombardment was done as described above. Two d later, the bombarded leaves were cut into 5 mm × 5 mm sections and placed on solid L2NK-spectinomycin (300 μg/mL) plates. The green calli that formed were transferred to L2NK-spectinomycin (400 μg/mL) plates and dissected monthly to allow continuous selection on L2NK-spectinomycin medium.

### PCR confirmation of putative transplastomic soybean embryos

DNA was extracted as described by Zhang et al. (2001) from a small piece of the resistant embryogenic tissue grown in the FG-spectinomycin (400 μg/mL) medium. The PCR was carried out with Taq DNA polymerase (GIBCO BRL) for 30 cycles at 94°C-45 sec, 55°C-45 sec and 72°C-0.5 min for primers C/D, 1.5 min for primers A/B or 3 min for primers E/F. As a control, we used the DNA isolated from the culture derived from the bombarded tissue but grown without selection. Three pairs of primers were used (Figure 1). Primer A, 5'-AACCTCAGGACCAGAAG TAG-3', is located in the coding region of soybean plastid atpβ gene, which is present in both wild type and transformed soybean cells. Primer B, 5'-CGGAACTGGTTC GATATC-3', is specific to the coding region of the *Chlamydomonas* rbcL. Likewise, the gene-specific primers C (5'-ATGATGGTCTGGACCCCGG-3') and D (5'-GCTAG CTTTACACGGAGCGCTTG-3') encompass the region of the *Chlamydomonas* rbcS gene. Therefore, with primers A/B or C/D, a 1.2-kb fragment containing the *Chlamydomonas* rbcL and soybean atpβ genes and a 0.4-kb fragment of the *Chlamydomonas* rbcS will be amplified only in the transplastomic soybean cells, respectively. To assess whether the transformed embryo was homoplastomic, we used another pair of primers common to both wild type and transformed soybean cells. Primer E, 5'-ACCCTT GGGGTAACGCTCCAGGTGCTG-3', is located in the coding region of the *Chlamydomonas* rbcL, which is very similar (24 bases identical out of 27 bases) to the sequence of soybean rbcL gene. Primer F, 5'-TTCATTAAGACTATA TTTTTC-3', is in the noncoding region of soybean rbcL. PCR with primers E/F would amplify a 0.7-kb fragment for the non-transformed wild type soybean cells and a 3.1-kb fragment in the transplastomic tissue.

## Results and Discussion

We attempted to transform the plastids of soybean leaf tissue and the PA cells, even though plants cannot be regenerated from these cells, to test the experimental methods. Based on our electron microscopic observations of the plastid size in the cultured soybean cells (Zhang et al., 2001), we chose gold particles that were 0.6 μm in diameter as the DNA carrier. The PA cultures bombarded with the transformation vector pZVII did not survive the initial selection with 200 μg/mL of spectinomycin and all cultures were dead within four weeks (Table 1). We found that the PA cells were very susceptible to mechanical handling and wounding. The PA cells bombarded with gold particles without DNA coating at different rupture pressures did not recover well after being returned to the $KN^0$ medium and in most cases the cells eventually died.

A total of 93 young leaves (ca. 5 cm in diameter) from greenhouse-grown plants were also bombarded. While most leaf sections became bleached on spectinomycin medium and died, two resistant green calli formed and grew well on L2NK with 400 μg/mL spectinomycin. After six months of selection, samples of the calli were subjected to Southern and Northern blot analyses using the aadA

gene as probe. No hybridizing signals were detected for either DNA or RNA blots (data not shown), indicating that these calli were not aadA gene-integrated transformants, but were apparently spontaneous plastid mutants, resistant to spectinomycin. Such mutants have been selected before, as for example, with tobacco (Fromm et al., 1987) and *Solanum nigrum* (Kavanagh et al., 1994).

We also attempted to transform soybean embryogenic tissues because these tissues are capable of regenerating into plants. The embryogenic suspension cultures (clumps about 1 mm in diameter) were bombarded with pZVII vector DNA-coated gold particles. Under spectinomycin selection, untransformed tissues gradually bleached and disintegrated while resistant tissue became bright green and grew. From 984 bombarded samples (a total of about 100,000 embryogenic clumps), only three resistant clumps were recovered (Table 1). One of the resistant clumps proliferated sufficiently in FG-spectinomycin medium (3 months after bombardment) so that a sample of the tissue could be used for DNA isolation for testing by PCR. When PCR was carried out to identify the integration of the transgene, a 1.2-kb fragment was amplified with primers A/B only in the DNA from the resistant embryogenic tissue (Figure 1B), which represents a DNA region flanking the soybean plastid atp$\beta$ gene and the *Chlamydomonas* rbcL gene (Figure 1A). Primers C/D identified a 0.4-kb *Chlamydomonas* rbcS coding sequence in the resistant tissue, whereas no amplified product was detected in the untransformed tissue (Figure 1B). To determine whether all copies of plastid genome were transformed (homoplastomy), we employed PCR with primers E/F. As shown in Figure 1B, a 0.7-kb fragment was detected in the wild type tissue, which flanks the region between rbcL and its 3'-noncoding sequence. For the resistant embryogenic tissue, a 3.1-kb DNA fragment was amplified, as expected for the insertion of aadA and *Chlamydomonas* rbcS genes along with their respective promoters and terminator sequences. However, a 0.7-kb fragment was also detected in this sample, suggesting the presence of copies of the wild type plastid genome. This indicates that transplastomic integration of the *Chlamydomonas* rbcL and rbcS genes had occurred in the selected tissue, but that not all plastid copies were transformed so the tissue was heteroplastomic. Further selection would be necessary to achieve homoplastomy, but none of the resistant embryos survived the subsequent proliferation and differentiation protocols so that further studies could not be carried out.

To our knowledge, plastid transformation has been reported in four species of higher plants-tobacco (Svab et al., 1993), rice (Khan and Maliga, 1999), *Arabidopsis* (Sikdar et al., 1998) and potato (Sidorov et al., 1999). However, only in the case of tobacco have fertile homoplastomic plants been obtained. In *Arabidopsis*, one transplastomic line was recovered from 100 bombarded samples, but none of the regenerated plants was fertile (Sikdar et al., 1998). The regenerated transplastomic rice plants were heteroplastomic (Khan and Maliga, 1999), while homoplastomic potato lines were obtained (Sidorov et al., 1999). However, information concerning the fertility and the fate of the transgenes in progeny of the transformed rice and potato plants was not reported.

There have been attempts to genetically engineer tobacco Rubisco by chloroplast transformation that resulted only in poor photosynthetic performance (Kanevski and Maliga, 1994; Kanevski et al., 1999; Whitney et al., 1999). In our efforts to improve the photosynthetic properties of a important crop plant, we attempted to modify the soybean Rubisco enzyme by plastid transformation. However, an extremely low transformation frequency was found with soybean embryogenic tissue since only one transplastomic event was identified in 984 bombardments. In contrast, we routinely obtain up to 10 transplastomic tobacco lines in 100 bombarded leaf samples (data not shown). We did not succeed in transforming either soybean leaf tissue or the PA cells, which may in part be due to the low number of bombardments attempted with these tissues, 93 and 30, respectively, and the

**Table 1.** Recovery of spectinomycin resistant tissues from bombarded leaves, photoautotrophic suspension cells (PA) and embryogenic tissues (EM) of soybean.

| | Leaf (5 cm diameter) | PA (fresh weight, mg) | EM (1 mm clumps) |
|---|---|---|---|
| Amount of target tissue per bombardment | 1 | ~100 | ~100 |
| Total number of bombardments | 93 | 30 | 984 |
| Number of resistant lines | 2 | 0 | 3 |
| Number of plastid transformants | 0 | | 1 |
| Shoot regeneration | | | 0 |

extreme susceptibility of the PA cells to the bombardment process.

Our PCR results that show the correct fragment sizes for transplastomic and untransformed lines (Figure 1B) are not likely to be an artifact due to plasmid contamination caused by the adherence of the plasmid DNA to the bombarded tissue. During the 3 months of selection, 12 medium changes were carried out. Whereas the tissue resistant to spectinomycin was shown to be PCR positive, the tissue that was bombarded with the same plasmid but grown in a medium without spectinomycin was PCR negative with all three primer sets used (Figure 1B). The results with primer set E/F show that the transplastomic 3.1-kb band is relatively high in intensity, while the intensity of the 0.7-kb band from the wild type plastid genome still present in the heteroplastomic line is decreased in comparison to the untransformed line.

The low number of plastids per cell and the low rate of photosynthesis in the embryogenic culture (Zhang et al., 2001) might contribute to the low transformation efficiency. This is further compounded by a relatively low rate of embryo proliferation and plant regeneration. All these factors contribute to the difficulty of plastid transformation in soybean. It is apparent that many obstacles need to be overcome such as a limited choice of regenerable target tissues and selectable markers, a low frequency of transformation, the poor regeneration of embryogenic tissue, and possible improvement of vector design and delivery, before plastid transformation technology can be readily applied to soybeans (Zhang et al., 2001).

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