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ORIGINAL PAPER

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Marker free transgenic plants: engineering the chloroplast genome without the use of antibiotic selection

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Abstract Chloroplast genetic engineering offers several advantages over nuclear transformation including high levels of gene expression and gene containment. However, a consequence of placing a transgene in the chloroplast genome is that the antibiotic resistance genes used as selectable markers are highly amplified. Engineering genetically modified (GM) crops without the use of antibiotic resistance genes should eliminate the potential risk of their transfer to the environment or gut microbes. Therefore, the betaine aldehyde dehydrogenase (BADH) gene from spinach was used in this study as a selectable marker. The selection process involves conversion of toxic betaine aldehyde (BA) by the chloroplast BADH enzyme to non-toxic glycine betaine, which also serves as an osmoprotectant. Chloroplast transformation efficiency was 25-fold higher in BA selection than with spectinomycin. In addition, rapid regeneration was obtained. Transgenic shoots appeared within 12 days in 80% of leaf disks (up to 23 shoots per disk) under BA selection compared to 45 days in 15% of disks (1 or 2 shoots per disk) under spectinomycin selection. Southern blots confirmed stable integration of foreign genes into all of the chloroplast genomes (~10,000 copies per cell) resulting in homoplasmy. Transgenic tobacco plants showed 15- to 18-fold higher BADH activity at different developmental stages than untransformed controls. Transgenic plants were morphologically indistinguishable from untransformed plants and the introduced trait was inherited stably in the subsequent generation. This is the first report of genetic engineering of the higher plant chloroplast genome without the use of antibiotic selection. The use of naturally occurring genes in spinach for selection, in addition to gene containment, should ease public concerns regarding GM crops.

Key words Antibiotic free selection · Clean-gene technology · Plastid transformation · drought tolerance · Osmoprotection

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Introduction

Most transformation techniques co-introduce a gene that confers antibiotic resistance, along with the gene of interest to impart a desired trait. Regenerating transformed cells in antibiotic-containing growth media permits selection of only those cells that have incorporated the foreign genes. Once transgenic plants are regenerated, antibiotic resistance genes serve no useful purpose but they continue to produce their gene products. One of the primary concerns of genetically modified (GM) crops is the presence of clinically important antibiotic resistance gene products in transgenic plants that could inactivate oral doses of the antibiotic (reviewed by Puchta 2000; Daniell 1999a). Another concern is that the antibiotic resistance genes could be transferred to pathogenic microbes in the gastrointestinal tract or soil rendering them resistant to treatment with such antibiotics. Antibiotic resistant bacteria are one of the major challenges of modern medicine. In Germany, GM crops containing antibiotic resistance genes have been banned from release (Peerenboom 2000). However, several approaches are currently available to eliminate antibiotic resistance genes from nuclear transgenic crops (Puchta 2000).

Chloroplast genetic engineering is emerging as an alternative new technology to overcome some of the environmental concerns of nuclear genetic engineering (reviewed by Bogorad 2000). One common environmental concern is the escape of foreign genes from transgenic crop plants to their weedy relatives, through pollen or seed dispersal creating super weeds or causing

genetic pollution among other crops (Daniell 1999b). Keeler et al. (1996) have summarized valuable data on the weedy wild relatives of 60 important crop plants and potential hybridization between crops and wild relatives. Among 60 crops, only 11 do not have congeners and the rest of the crops have wild relatives somewhere in the world. In addition, genetic pollution among crops has resulted in several lawsuits and shrunk the European market of Canadian organic farmers (Hoyle 1999). Several major food corporations have required segregation of native crops from those "polluted" with transgenes. Two legislations have been submitted in the United States to protect organic farmers whose crops inadvertently contain transgenes via pollen drift (Fox 2000). Maternal inheritance of foreign genes through chloroplast genetic engineering is highly desirable in such instances where there is potential for out-cross among crops or between crops and weeds (Daniell et al. 1998; Daniell 1999c; Scott and Wilkinson 1999).

Yet another concern in the use of nuclear transgenic crops expressing the *Bacillus thuringiensis* (Bt) toxins is the sub-optimal production of toxins resulting in an increased risk of pests developing Bt resistance. Plant-specific recommendations to reduce the development of Bt resistance include increasing Bt expression levels (high dose strategy), expressing multiple toxins (gene pyramiding), or expressing the protein only in tissues highly sensitive to damage (tissue specific expression). All three approaches are attainable through chloroplast transformation (Daniell 1999c). For example, hyper-expression of several thousand copies of a novel Bt gene via chloroplast genetic engineering, resulted in 100% mortality of insects that are up to 40,000-fold resistant to other Bt proteins (Kota et al. 1999). Another hotly debated environmental concern expressed recently is the toxicity of transgenic pollen to non-target insects, such as the Monarch butterflies (Hodgson 1999; Losey et al. 1999). Although pollen from a few plants shown to exhibit maternal plastid inheritance contains metabolically active plastids, the plastid DNA itself is lost during the process of pollen maturation and hence is not transmitted to the next generation (reviewed in Heifetz 2000; Bock and Hagemann 2000). Lack of insecticidal protein in transgenic pollen engineered via the chloroplast genome with the cry2 A gene has been demonstrated recently, even though chloroplasts in leaves contained as much as 47% CRY protein of the total soluble protein (De Cosa et al. 2000).

Despite these advantages, one major disadvantage with chloroplast genetic engineering in higher plants may be the utilization of the antibiotic resistance genes as the selectable marker to confer streptomycin/spectinomycin resistance. Initially, selection for chloroplast transformation utilized a cloned mutant 16 S rRNA gene that does not bind the antibiotic and this conferred spectinomycin resistance (Svab et al. 1990). Subsequently, the *aadA* gene product that inactivates the antibiotic by transferring the adenyl moiety of ATP to spectinomycin/streptomycin was used (Svab and Maliga 1993). These antibiotics are commonly used to control bacterial infection in humans and animals. The probability of gene transfer from plants to bacteria living in the gastrointestinal tract or soil may be enhanced by the compatible protein synthetic machinery between chloroplasts and bacteria, in addition to the presence of thousands of copies of the antibiotic resistance genes per cell. Also, most antibiotic resistance genes used in genetic engineering originate from bacteria. Therefore, the betaine aldehyde dehydrogenase (BADH) gene from spinach has been used in this study as a selectable marker. This enzyme is present only in chloroplasts of a few plant species adapted to dry and saline environments (Rathinasabapathy et al. 1994; Nuccio et al. 1999). The selection process involves conversion of toxic betaine aldehyde (BA) by the chloroplast BADH enzyme to non-toxic glycine betaine, which also serves as an osmoprotectant (Rathinasabapathy et al. 1994). In *Chlamydomonas*, chloroplast transformation was first accomplished by complementation of deletion mutants (Boynton et al. 1988). Recycling of selectable markers using direct repeat sequences has also been successful in *Chlamydomonas* (Fischer et al. 1996). However, none of these approaches have been extended to higher plant chloroplast transformation so far.

Materials and methods

Plasmid vectors and gene expression in *Escherichia coli*

The pLD-BADH plasmid was constructed as described in the Results section and was transformed into the *E. coli* strain XL-1 Blue and grown in Terrific Broth (Guda et al. 2000) in the presence of ampicillin (100 μg/ml) at 37 °C for 24 h. The BADH gene expression was tested in *E.coli* cell extracts by enzyme assays before proceeding with bombardment.

Bombardment and regeneration of chloroplast transgenic plants

Tobacco (*Nicotiana tabacum* var. Petit Havana) was grown aseptically by germination of seeds in MSO medium. This medium contains MS salts (4.3 g/l), B5 vitamin mixture (myo-inositol, 100 mg/l; thiamine-HCl, 10 mg/l; nicotinic acid, 1 mg/l; pyridoxine-HCl, 1 mg/l), sucrose (30 g/l) and phytagar (6 g/l) at pH 5.8. Fully expanded, dark green leaves of about 2- month-old plants were used for bombardment.

Leaves were placed abaxial side up on Whatman No. 1 filter papers laying on the RMOP medium (Daniell 1993) in standard petri plates (100 × 15 mm) for bombardment. Microprojectiles were coated with plasmid DNA (pLD-BADH) and bombardments were carried out with the biolistic device PDS1000/He (Bio-Rad) as described by Daniell (1997). Following bombardment, petri plates were sealed with parafilm and incubated at 24 °C under 16 h photoperiod. Two days after bombardment, leaves were chopped into small pieces of ~5 mm^2 in size and placed on the selection medium (RMOP containing 500 μg/ml of spectinomycin dihydrochloride or 5–10 mM BA) with abaxial side touching the medium in deep (100 × 25 mm) petri plates. The regenerated resistant shoots were chopped into small pieces (~2mm^2) and subcloned into fresh deep petri plates containing the same selection medium. Resistant shoots from the second culture cycle were transferred to the rooting medium (MSO medium supplemented with IBA, 1 mg/l containing appropriate selectable marker). Rooted plants were

transferred to soil and grown at 26 °C under 16 h photoperiod for further analysis.

Polymerase chain reaction

In order to conduct PCR analyses in transgenic plants, total DNA from non-bombarded and transgenic plants was isolated using the DNeasy kit (Quiagen, Valencia, Calif.). PCR was performed in a total volume of 50 µl containing approximately 10 ng of template DNA and 1 µM of each primer in a mixture of 300 µM of each deoxynucleotide (dNTPs), 200 mM Tris (pH 8.8), 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100, 1 mg/ml nuclease-free BSA and 1 or 2 units of Taq Plus polymerase (Stratagene, La Jolla, Calif.). PCR was carried out in the Perkin Elmer's GeneAmp PCR system 2400, by subjecting the samples to 94 °C for 4 min and 30 cycles of 94 °C for 1.5 min, 65 °C for 1.5 min, 72 °C for 4 min followed by a 72 °C step for 10 min. PCR products were analyzed by electrophoresis on 0.8% agarose gels. Chloroplast transgenic plants containing the BADH gene were moved to a second round of selection in order to achieve homoplasmy.

Southern blot analysis

Total DNA was extracted from leaves of transformed and wild type plants using the DNeasy kit (Qiagen). Total DNA was digested with *Bgl*II, electrophoresed on 0.7% agarose gels and transferred to nylon membranes (Micron Separation Inc., Westboro, Mass.). The probes were labeled with ^{32}P-dCTP using the random-primed procedure (Probequant; Amersham, Piscataway, N.J.). Prehybridization and hybridization steps were carried out at 42 °C using the Stratagene Quick-Hyb solution. Blots were soaked in a solution containing 2× SSC and 0.5% SDS for 5 min followed by transfer to 2× SSC and 0.1% SDS solution for 15 min at room temperature. Blots were then incubated in hybridization bottles containing 0.1× SSC and 0.5% SDS solution for 30 min at 37 °C followed by another step at 68 °C for 30 min, with gentle agitation. Finally, blots were washed in 0.1× SSC solution, dried and exposed to X-ray film.

BADH assay

Extraction and assay for BADH was done as described by Weretilnyk et al. (1989) except that crude extracts were used for enzyme assays without ammonium sulfate fractionation and purification. Leaf material (1–2 g) was ground in a mortar at 0–4 °C (on ice) in 2 ml of extraction buffer [50 mM Hepes-KOH (pH 8.0),1 mM EDTA, 20 mM sodium metabisulfite, 10 mM sodium borate, 5 mM ascorbic acid and 5 mM DTT].The extract was centrifuged for 10 min at 10,000*g* (4 °C).The supernatant was desalted using small columns of Sephadex G-25 (Amersham Pharmacia Biotech) equilibrated with the assay buffer [50 mM Hepes-KOH (pH 8.0), 1 mM EDTA and 5 mM DTT]. NAD reduction was assayed spectrophotometrically at 340 nm. Assays were done in 1 ml volume containing 50 mM Hepes-KOH (pH 8.0), 5 mM DTT, 1 mM EDTA and 1 mM NAD+. The reaction was started by the addition of 1 mM BA at 22 °C. For *E. coli*, cultures (100 ml) were centrifuged and suspended in minimal volume. Cells were sonicated and desalted like the leaf sample and used for the assays.

Results and discussion

Chloroplast integration vector and *E. coli* expression

The universal chloroplast vector pLD-CtV targets integration of the foreign genes into the inverted repeat (IR) region of the tobacco chloroplast genome (Daniell et al. 1998; Guda et al. 2000). The flanking chloroplast DNA sequence of the universal vector is highly conserved among higher plants and, therefore, could be used to transform chloroplast genomes of higher plants. The vector pLD-BADH was constructed by generating a PCR product using a spinach cDNA clone (kindly provided by Dr. Andrew Hanson, University of Florida, Gainesville) as the template. The 5′ primer also included the chloroplast optimal ribosome binding site (GGAGG). The PCR product was subcloned into the *Eco*R1 site of pLD-CtV, resulting in pLD-BADH (Fig. 1). BADH is one of the few proteins targeted to the chloroplast that lacks a definite transit peptide (Rathinasabapathy et al. 1994). The authors suggest that information for transport may be contained within the mature protein. Even if a transit peptide was present, it should be cleaved in the stroma by the stromal processing peptidase (Keegstra and Cline 1999). Furthermore, nuclear encoded cytosolic proteins with transit peptides have been successfully expressed within chloroplasts and found to be fully functional (Daniell et al. 1998). Therefore, there was no need to delete any transit peptide.

The universal vector, pLD-BADH (Fig. 1) integrates the *aadA* and BADH genes into the 16S-23S-spacer region of the chloroplast genome. Expression cassettes of the chloroplast integration vector contain the chimeric *aadA* gene and the BADH gene driven by the constitutive 16 S rRNA promoter and regulated by the 3′ untranslated region of the plastid *psbA* gene. The chimeric *aadA* gene encoding aminoglycoside 3′-adenyltransferase confers spectinomycin resistance in chloroplasts enabling selection of the transformants on spectinomycin dihydrochloride. On the other hand, BADH converts the toxic BA in cells to glycine betaine (which is used as an osmoprotectant by drought tolerant plants) and when present, this pathway is compartmentalized within chloroplasts (Nuccio et al. 1999). To facilitate translation of the dicistronic mRNA, independent Shine-Dalgarno (SD) sequences were provided to the *aadA* and BADH genes upstream of the initiation codons. In order to accurately compare transformation efficiency of both selectable markers under identical bombardment and transformation conditions, *aadA* and BADH genes were inserted into the same vector, at the same site. Bom-

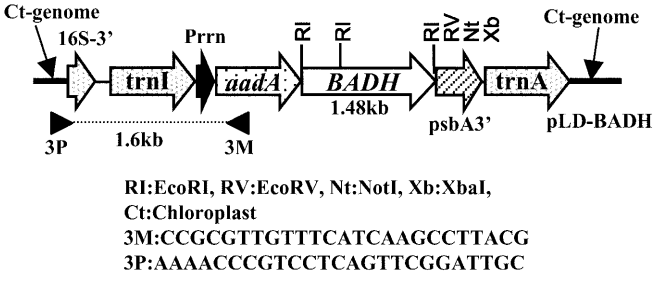

Fig. 1 Chloroplast vector pLD BADH. Primer 3P lands on the native chloroplast genome (in the 5′ end region of 16 S rDNA gene). 3M lands on the *aadA* gene generating a 1.6 kb fragment. Restriction enzyme cut sites are located on the map

barded leaves were treated in identical manner except for the addition of selection reagent.

In *E.coli*, the level of expression by the chloroplast *Prrn* promoter is equivalent to that of the highly efficient T7 promoter and both systems have highly compatible protein synthetic machinery (Brixey et al. 1997). Therefore, before proceeding with bombardment, the BADH enzyme activity was tested in untransformed cells and cells transformed with pLD-BADH, a high copy number plasmid (Fig. 2). Crude sonic extracts isolated from transformed cells showed 3- to 5-fold more BADH activity than the untransformed control, confirming that the expression cassette is fully functional. This result also suggests that codon preference of the nuclear BADH gene is compatible with expression in the prokaryotic chloroplast compartment.

Selection and regeneration of homoplasmic transgenic plants

Fully expanded, green leaves of *Nicotiana tabacum* var. Petit Havana were transformed with the chloroplast integration and expression vector pLD-BADH, by the biolistic process as described by Daniell (1993, 1997). The entire process of regeneration, starting from bombardment until transfer to soil, takes about 3–6 months for spectinomycin selection and 2–3 months for BA selection. Figure 3 and Table 1 show differences between the two selection processes. Under spectinomycin selection, leaf disks continued to grow but pigments were bleached; resistant clones formed green shoots in about 45 days (Fig. 3B). On the other hand, under BA selection, growth of the leaf disks was completely inhibited and photosynthetic pigments were degraded (Fig. 3G-1); resistant clones formed green shoots within 12 days (Fig. 3E). Leaf disks in Fig. 3 under BA selection appear partially green because they were photographed 12 days after the initiation of the selection process whereas the disks photographed on spectinomycin were 45 days after initiation of the selection process. In spite of the short period of selection one leaf disk was almost bleached (Fig. 3D) and all of them were killed after 30 days. Under 10 mM BA selection, control untransformed samples were killed (turned black, 3G-1) whereas transgenic leaves produced new shoots (Fig. 3G, 2–4).

When the leaf disks were selected for spectinomycin resistance, only 15% of the disks responded and an average of one resistant shoot per plate was observed after 45 days. From each callus, all resistant shoots are considered to represent an individual clone. Under BA selection 80% of the disks responded and an average of 25 resistant shoots per plate was observed. Responding leaf disks formed one or two resistant shoots under spectinomycin selection whereas under BA selection, as many as 23 shoots were observed from a single leaf disk. Overall, 10 resistant shoots were regenerated from ten bombardments under spectinomycin selection while more than 150 shoots were recovered from six bombardments under BA selection. Therefore, the efficiency of transformation is 25-fold higher in BA selection than in spectinomycin selection. Additionally, the latter procedure results in rapid regeneration.

Integration of a foreign gene into the chloroplast genome was confirmed by PCR screening of chloroplast transformants (Fig. 4). Primers were designed to eliminate mutants, nuclear integration and to determine whether the integration of foreign genes had occurred in the chloroplast genome at the directed site by homologous recombination. The strategy employed to distinguish between nuclear and chloroplast transgenic plants was to land one primer (3P) on the native chloroplast genome adjacent to the point of integration and the second primer (3 M) on the *aadA* gene (Fig. 1). This primer set generated 1.6 kb PCR product in chloroplast transformants (Fig. 4). Because this product cannot be obtained in nuclear transgenic plants, the possibility of nuclear integration can be eliminated. PCR screening for chloroplast transformants after the first culture cycle showed that 11 out of 15 BA resistant clones integrated foreign genes into the chloroplast genome. The rest of the resistant shoots may be either escapes or nuclear transformants. Hence, only PCR positive clones were advanced to further steps of regeneration. In contrast, nearly 60% of the spectinomycin resistant clones were mutants. Other laboratories have recently reported as high as 90% mutants among spectinomycin resistant clones (Eibl et al. 1999; Sidorov et al. 1999).

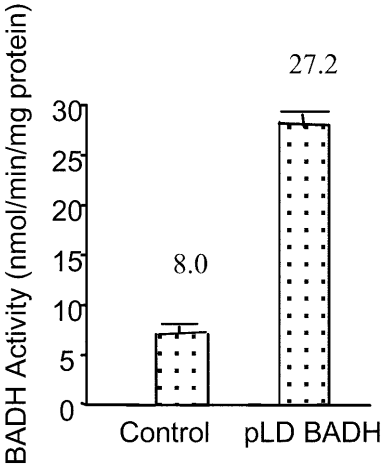

Fig. 2 BADH enzyme activity in *Escherichia coli*. Cells harvested from overnight grown cultures were resuspended in a minimal volume of the assay buffer. Sonicated cell homogenate was desalted in G-25 columns and 50 μg total protein was used for each assay. NAD+ dependent BADH enzyme was analyzed for the formation of NADH by increase in absorbency at 340 nm

Determination of chloroplast integration, homoplasmy and copy number

Southern blot analysis was performed using total DNA isolated from transgenic and wild type tobacco leaves.

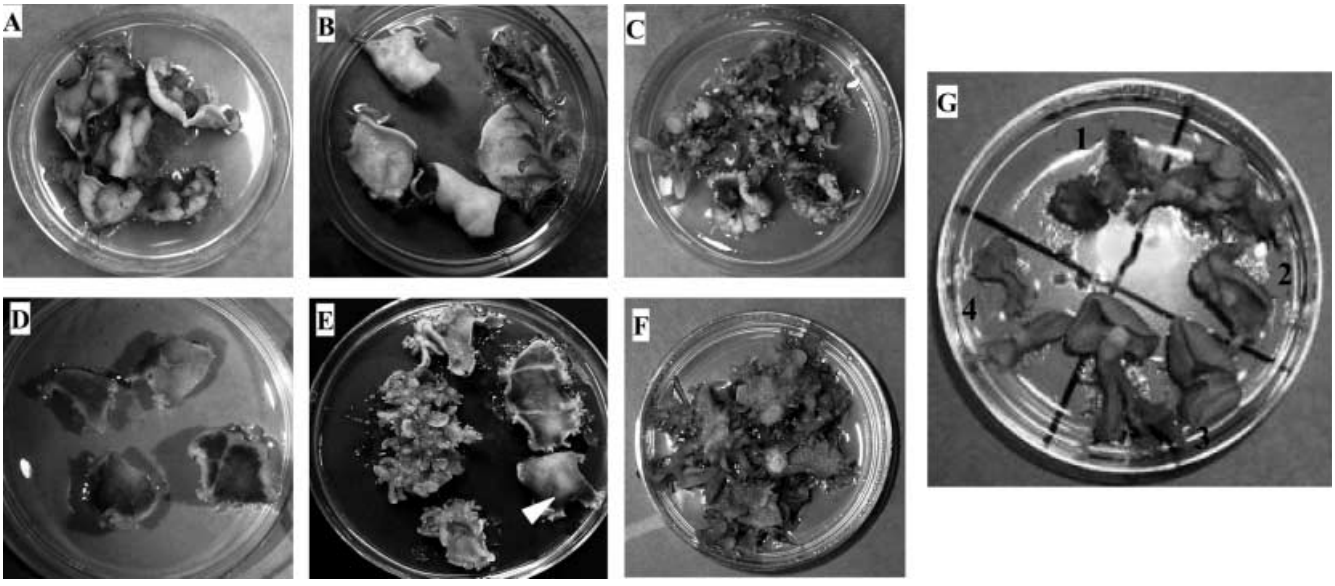

Fig. 3A–G Comparison of betaine aldehyde and spectinomycin selection. **A** *N. tabacum* Petit Havana control in RMOP medium containing spectinomycin after 45 days. **B** Bombarded leaf disks selected on spectinomycin in RMOP medium after 45 days. **C** Spectinomycin resistant clones cultured again (second round) to obtain homoplasmy. **D** Petit Havana control in RMOP medium containing betaine aldehyde after 12 days of culture. **E** Bombarded leaf disks selected on betaine aldehyde in RMOP medium after 12 days of culture; *arrow* indicates unbombarded leaf disk as control. Note that 23 shoots are formed on a disk selected on betaine aldehyde against 1–2 shoots per disk on spectinomycin. **F** Betaine aldehyde resistant clones cultured again (second round) to obtain homoplasmy. **G** Selection on 10 mM betaine aldehyde of untransformed (1) and transgenic (2–4) leaf disks. Note shoots from transgenic leaf disks and death of untransformed leaf disk

Table 1 Comparison of spectinomycin and betaine aldyhyde as the selectable marker for the first round of selection

Selectable marker	Plate no.	Total no. of leaf disks	No. of responding leaf disks	Total no. of shoots/ plate
BADH	1	3	3	43
	2	6	4	23
	3	11	9	33
	4	7	6	19
	5	6	4	16
	6	9	7	18
Spectinomycin	1	5	0	0
	2	5	0	0
	3	5	3	3
	4	5	2	2
	5	5	0	0
	6	5	1	1
	7	5	1	2
	8	5	1	2
	9	5	0	0
	10	5	0	0
Control		5	0	0

Total DNA was digested with a suitable restriction enzyme. Presence of a *Bgl*II cut site at the 3′ end of the flanking 16 S rRNA gene and the *trnA* intron allowed excision of predicted size fragments in the chloroplast transformants and untransformed plants. To confirm foreign gene integration and homoplasmy, individual blots were probed with the flanking chloroplast DNA sequence (probe 1, Fig. 5 A). In the case of the BADH integrated plastid transformants, the border sequence hybridized with a 7.29 kbp fragment while it hybridized with a native 4.47 kbp fragment in the untransformed plants (Fig. 5B). The copy number of the integrated BADH gene was also determined by establishing homoplasmy in transgenic plants (Daniell et al. 1998; Guda et al. 2000). Tobacco chloroplasts contain about 10,000 copies of chloroplast genomes per cell. If only a fraction of the genomes were transformed, the copy number would be less than 10,000. By confirming that the BADH integrated genome is the only one present in transgenic plants, it could be established that the BADH gene copy number could be as many as 10,000 per cell.

DNA gel blots were also probed with the BADH gene coding sequence (P2) to confirm specific integration into the chloroplast genomes and eliminate transgenic plants that had foreign genes also integrated into the nuclear genome. In the case of the BADH integrated plants, the BADH coding sequence hybridized with a 7.29 kbp fragment which also hybridized with the border sequence in plastid transformant lines (Fig. 5B). This shows that the BADH gene was integrated only into the chloroplast genome and not the nuclear genome in transgenic lines examined in this blot. This also confirms that the tobacco transformants integrated the intact gene expression cassette into the chloroplast genome and that no internal deletions or loop outs during integration occurred via homologous recombination.

BADH enzyme activity

In higher plants accumulation of osmoprotectants during salinity and drought stress is a common phenome-

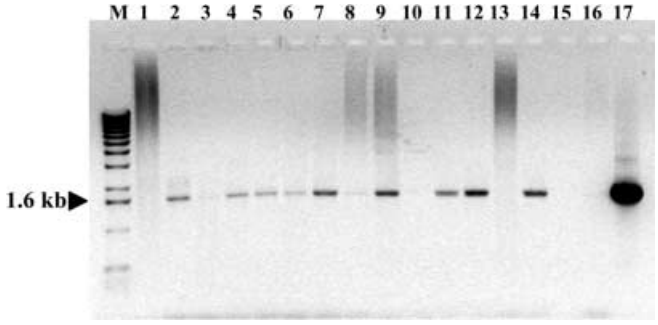

Fig. 4 PCR analysis of DNA extracted from transformed plants run on a 0.8% agarose gel. *Lane M* 1 kb ladder, *lane1* untransformed Petit Havana control, *lane 17* is positive control and *lanes 2–16* are transgenic clones. Except *lanes 10*, *13*, *15 and 16* all other lanes show the integration of *aadA* gene into the chloroplast genome

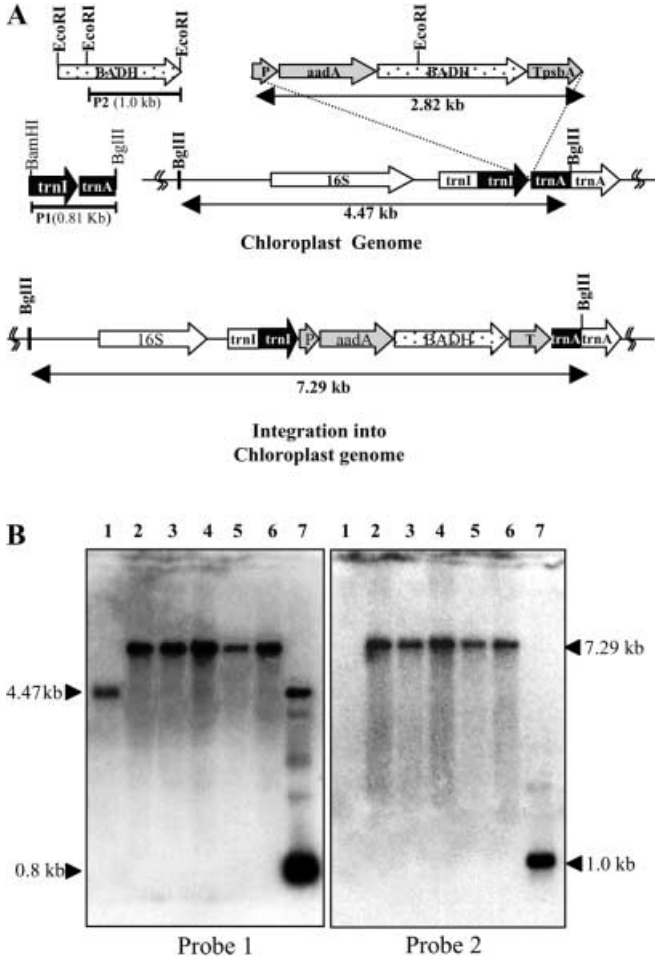

Fig. 5 A,B Southern analysis of transgenic plants. **A** Probe P1 was used to confirm chloroplast integration of foreign genes. The 0.81 kb fragment was cut with *Bam*HI and *Bgl*II contains the flanking sequence used for homologous recombination. Untransformed control plants should generate 4.47 kb fragment and transformed plants should generate a 7.29 kb fragment. **B** *Lanes 1* Untransformed Petit Havana; *lanes 7* pLD-BADH plasmid DNA or purified 1.0 kb *Eco*RI BADH gene fragment. *Lanes 2–6* of transgenic plants. Probe (P2) was used to confirm the integration of BADH gene

non in their metabolic adaptation. Osmoprotectants help to protect plant organelles from osmotic shock as well as the cellular membranes from damage during stress (Nuccio et al. 1999). Among the osmoprotectants, glycine betaine is the most effective and commonly present in a few families, including Chenopodiaceae and Poaceae. However, most of the crop species including tobacco do not accumulate glycine betaine. Since synthesis and localization of glycine betaine is compartmentalized in chloroplasts, engineering the chloroplast genome for glycine betaine synthesis may provide an added advantage for chloroplast transgenic plants. BADH converts toxic BA to non-toxic glycine betaine, the second step in the formation of glycine betaine from choline. By analyzing BADH enzyme activity, the expression of introduced BADH gene can be monitored. Since BADH is NAD+ dependent, enzyme activity is analyzed for the formation of NADH. The reaction rate is measured by an increase in absorbency at 340 nm resulting from the reduction of NAD+.

BADH enzyme activity was assayed in crude leaf extracts of wild type and transgenic plants. Unlike previous reports, no purification with ammonium sulfate was necessary in order to perform the BADH assay. Crude extracts from chloroplast transgenic plants showed elevated activity (15-to 18-fold) compared to the untransformed tobacco (Fig. 6). The wild type tobacco showed low endogenous activity as reported previously (Rathinasababathy et al. 1994). BADH enzyme activity was investigated from young (top 3–4 leaves), mature (large, well developed), developing leaves (in between young and mature) and bleached old leaves from transgenic plants. Crude leaf extracts from

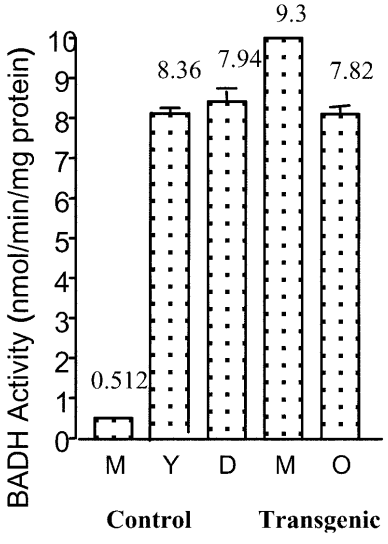

Fig. 6 BADH enzyme activity in transgenic tobacco plant leaves of different ages . Proteins were extracted from 1- to 2-g leaves. Extracts were centrifuged at 10,000*g* for 10 min and the resulting supernatant was desalted in small G-25 columns, and tested for assay (50 µg protein per assay). NAD+ dependent BADH enzyme was analyzed for the formation of NADH. *Y*, *D*, *M* and *O* represent young, developing, mature and old leaves, respectively

different developmental stages of the same transgenic plant showed differential activity with the most activity observed in mature leaves (18-fold over control) and least activity in older leaves (15-fold over control, as seen in Fig. 6). Unlike nuclear transgenic lines, crude extracts from different chloroplast transgenic lines did not show significant variation in BADH activity (data not shown).

Expression of BADH and resultant accumulation of glycine betaine did not result in any pleiotropic effects; transgenic plants are morphologically indistinguishable from control untransformed plants (Fig. 7). Transgenic plants grew normally, flowered and set seeds. However, a detailed characterization of T_1 transgenic lines is in progress to fully examine the consequence of these transformation events. Germination of seeds from untransformed plants in the presence of spectinomycin resulted in complete bleaching whereas seeds from the chloroplast transgenic plants germinated and grew normally (Fig. 8). Because untransformed seeds germinated in very high concentrations of BA (10–15 mM), no comparison between control and transgenic seeds could be made during germination on BA. This may be due to the presence of an active endogenous BADH or similar

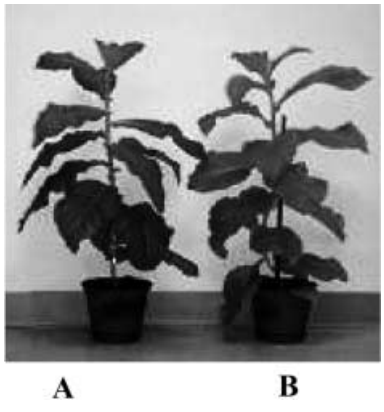

Fig. 7 Phenotypes of control (**A**) and chloroplast transgenic (**B**) plants

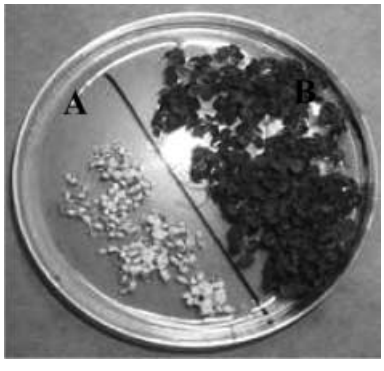

Fig. 8 Germination of control untransformed (**A**) and chloroplast transgenic (**B**) seeds on MS medium containing 500 μg/ml spectinomycin

enzymatic activity in non-green plastids during germination. These results demonstrate that the introduced trait is stably inherited in the subsequent generation and that BA selection is safe to use because of the lack of pleiotropic effects.

Thousands of antibiotic resistant genes are present in each cell of chloroplast transgenic plants. This fact, together with the use of the most commonly used antibiotics in the selection process, makes it important to develop a chloroplast genetic engineering approach without the use of antibiotics. Comparative studies on selection of bombarded leaves on spectinomycin and BA reveal that shoot regeneration could be accomplished in a much shorter time with selection on BA. Because the 16 S rRNA gene can undergo mutation, selection on spectinomycin results in a large number of mutants. BA selection, however, does not result in such mutations. Nevertheless, a few escapes or nuclear transformants were observed as with other selectable markers. When the chloroplast transformation system was developed, it was hypothesized that the transformation process would only be possible under non-lethal selection (Svab and Maliga 1993). Non-lethal selection was defined in the chloroplast transformation literature as lack of suppression of growth on the selection medium and that this was an absolute requirement for plastid transformation (Svab et al. 1990). It is known that accumulation of BA is toxic and lethal to plant cells (Rathinasabapathy et al. 1994). Results reported here confirm earlier observations that BA is toxic to plant cells and inhibits growth. Therefore, this investigation illustrates an important finding that non-lethal selection is not a requirement for chloroplast transformation. The only requirement is that the selection process be specific to chloroplasts. Indeed, spectinomycin is lethal to tissues other than tobacco; for example it was toxic to potato tissues and yet stable chloroplast transformants were recovered (Sidorov et al. 1999). Therefore, it is evident that chloroplast transformation does not require non-lethal selection. An added advantage of using BADH as a selectable marker is that it may confer drought/salt tolerance (Rathinasabapathy et al. 1994). Investigations are in progress to compare drought tolerance in BADH transgenic plants engineered via the nuclear and chloroplast genomes. Accomplishing genetic engineering without the use of antibiotic resistance genes but using genes that are naturally present in plants should ease public concerns on GM crops. In addition, higher efficiency of BA selection than spectinomycin should facilitate chloroplast transformation of many economically important crops, including cereals that are naturally resistant to spectinomycin.

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