

RESEARCH PAPER

Tobacco plastid transformation using the feedback-insensitive anthranilate synthase [α]-subunit of tobacco (ASA2) as a new selectable marker

Pierluigi Barone^{1,*†}, Xing-Hai Zhang² and Jack M. Widholm¹

¹ University of Illinois, Department of Crop Sciences, Edward R. Madigan Lab, 1201 W Gregory Dr, Urbana, IL 61801, USA

² Florida Atlantic University, Department of Biological Sciences, 777 Glades Road, Boca Raton, FL 33431, USA

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Abstract

Genetic engineering of chloroplasts normally requires the stable introduction of bacterial derived antibiotic or herbicide-resistance genes as selective markers. Ecological and health concerns have been raised due to the presence of such genes within the environment or the food supply. One way to overcome this issue is the use of plant genes able to confer a metabolic or developmental advantage to the transformed cells manipulating the plant's biosynthetic pathways. We explored the feasibility of using, for plastid transformation, the selection system based on the feedback-insensitive anthranilate synthase (AS) α -subunit gene of tobacco (ASA2) as a new selective marker and the indole analogue 4-methylindole (4MI) or the tryptophan analogue 7-methyl-DL-tryptophan (7MT) as the selection agents. An expression cassette containing Prn-ASA2 was effectively integrated into the region between *accD* and *ycf4* of the tobacco plastome by the biolistic process. Plastid transgenic plants were obtained on medium supplemented with 300 μ M 7MT or 4MI. Transplastomic plants showed normal phenotype and fertility and the resistance to the selection agents 7MT and 4MI was transmitted maternally. The plastid transformed lines also exhibited a higher level of AS enzyme activity that was less sensitive to Trp-feedback inhibition and, consequently, increased free Trp levels in leaves about 7-fold.

Key words: Anthranilate synthase, 7-methyl-DL-tryptophan, 4-methylindole-tryptophan, non-antibiotic selection, plastid transformation, selectable marker.

Introduction

The advantages of chloroplast genetic engineering are numerous and have been extensively reviewed (Bock, 2007; Lutz *et al.*, 2007; Verma and Daniell, 2007). They include the high-level accumulation of foreign proteins (Oey *et al.*, 2009), a lack of epigenetic effect and gene silencing, the possibility of gene stacking, and containment of the transgene due to the maternal inheritance of chloroplasts in most of the crop plants (Ruf *et al.*, 2007).

The successful recovery of a genetically stable transplastomic plant depends on the capability to selectively amplify the one or very few plastid genome (ptDNA) copies that are transformed in the initial biolistic experiment and

the key element for this process is the choice of selective markers.

There are two classes of plastid marker genes: 'primary selective markers' to be used for direct selection (*aadA*, *neo*, and *aphA-6*) and 'secondary selective markers' (*bar* or *CP4*) that are not suitable for direct selection if only a few ptDNA copies are transformed, but will allow selection when most of the ptDNA copies are transformed (Maliga, 2004; Lutz *et al.*, 2007). The 'primary selective markers' have bacterial origins and confer resistance to an antibiotic: the *aadA* gene confers resistance to spectinomycin and streptomycin (Svab and Maliga, 1993; Zoubenko *et al.*,

* Present address: Chromatin, Inc. 3440 South Dearborn St. Suite 280 Chicago, Illinois 60616, USA.

† To whom correspondence should be addressed: E-mail: pierluigibarone@gmail.com

Abbreviations: AS, anthranilate synthase; Trp, tryptophan; 7MT, 7-methyl-DL-tryptophan; 4MI, 4-methylindole; ptDNA, plastid genome. Published by Oxford University Press [on behalf of the Society for Experimental Biology] 2009.

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1994) while *neo* (Carrer *et al.*, 1993) and *aphA-6* (Huang *et al.*, 2002) confer resistance to kanamycin.

The ‘secondary selective markers’ are bacterial-derived as well and confer resistance to herbicides like phosphinothricin (*bar*) (Lutz *et al.*, 2001) or glyphosate (*CP4*) (Ye *et al.*, 2003).

Over the past several years, consumer and environmental groups have expressed ethical and biosafety concerns about the use of antibiotic- and herbicide-resistance genes derived from micro-organisms (Miki and McHugh, 2004). Therefore, strategies have been developed either (i) to excise the marker gene, once the selection is complete, in order to obtain a marker-free transplastomic plant or (ii) to use plant-derived markers.

Four protocols have been established to date for obtaining marker-free transplastomic plants: (i) homology-based excision via direct repeated sequences (Iamtham and Day, 2000); (ii) cotransformation–segregation (Carrer and Maliga, 1995); (iii) transient coinTEGRATION of the marker gene (Klaus *et al.*, 2004), and (iv) excision by phage site-specific recombinases (Corneille *et al.*, 2001). In the first two protocols the final transplastomic line is identified in a genetically unstable/segregating ptDNA population making it difficult to obtain the marker-free transplastomic plants. The third protocol is an antibiotic/phenotypic selection system that utilizes plastid mutants (e.g. knockout of a photosynthetic gene that produces a chlorophyll-deficient phenotype with pale-green leaves) as target material for chloroplast transformation. The need to produce the knockout mutant prior to the actual transformation makes the system not very convenient. The last protocol uses two different site-specific recombinases (*Cre* and *Int*) for plastid marker gene excision and, with both of them, the problem is the presence of potential pseudo sites in the ptDNA recognized by the enzyme.

Recently, a spinach (*Spinacia oleracea*)-derived selective marker betaine aldehyde dehydrogenase (BADH) gene has been used for plastid transformation in tobacco (Daniell

et al., 2001). However, these results have not been repeatable (Maliga, 2004; Whitney and Sharwood, 2008).

Anthranyl synthase (AS) catalyses the first reaction in the tryptophan (Trp) biosynthetic pathway by converting chorismate to anthranilate. Plant AS is a heterotetramer containing two large α -subunits and two small β -subunits (Romero *et al.*, 1995) and is feedback-regulated by the end-product, Trp, which binds to the α -subunit allosteric site (Bohlmann *et al.*, 1996; Li and Last, 1996). A naturally occurring feedback-insensitive α -subunit gene (*ASA2*) has been isolated from a tobacco suspension-culture cell line resistant to the toxic Trp analogue 5-methyltryptophan (5MT) (Song *et al.*, 1998). Trp analogues are toxic since they are able to mimic the specific feedback effect of Trp on the AS, therefore inhibiting Trp biosynthesis and causing Trp deficiency for protein synthesis. It has also been shown that plant cell cultures can convert various indole analogues to Trp analogues via the action of Trp synthase (Widholm, 1981). A feedback-sensitive AS enzyme fails to discriminate completely between its normal feedback inhibitor, Trp, and an analogue; hence if the feedback-insensitive *ASA2* is expressed then the cells are resistant to the analogues and can be selected for.

Previously (Barone and Widholm, 2008), the efficacy of the *ASA2* gene as a selective marker was described using either 4-methylindole (4MI) or 7-methyl-DL-tryptophan (7MT) as the selection agent for tobacco nuclear transformation and now the possibility of using this non-antibiotic, native plant gene selection system for tobacco plastid transformation is investigated.

Materials and methods

Plasmid vector

The vector pAST-IV (Fig. 1) as previously described by Zhang *et al.* (2001) contains a 1671 bp long modified

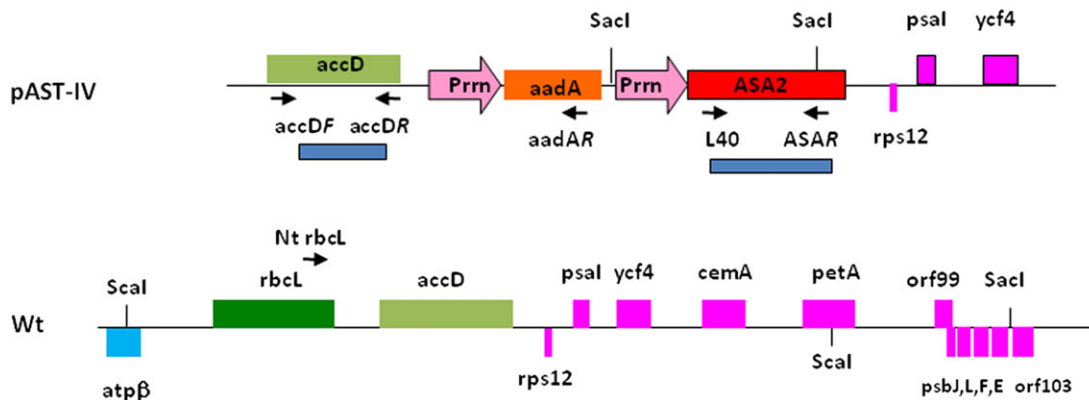


Fig. 1. Structure of the vector pASTIV used for tobacco transformation and its corresponding region of the wild-type tobacco plastid genome based on GenBank Z00044 and NC_001879. Relevant plastid genes and their orientation are presented. Prrn: plastid 16S rRNA operon promoter, *aadA*: spectinomycin resistance gene with the *Chlamydomonas* *rbcl* 3'-untranslated region (500 bp), *ASA2*, modified version of *ASA2* (1671 bp) including its termination sequence (204 bp). The locations of *SacI* and *Scal* used for Southern hybridization are shown. Location and orientation of PCR primers are indicated by arrows. *accD* probe (*accD* F–*accD* R) and *ASA2* probe (L40–ASAR) are shown. The figure size is not to scale.

version of the ASA2 gene (GenBank gi: 3348123) without a putative transit peptide and with an ASA2 3'-non-coding region (204 bp) as the termination sequence. The plasmid also has the *aadA* gene with the *Chlamydomonas rbcL* 3'-untranslated region. Both genes are driven by a chloroplast 16S rRNA promoter Prn (Goldschmidt-Clermont, 1991; Eibl *et al.*, 1999) and the site of insertion in the plastid genome is the intergenic region between *accD* and *ycf4* at nucleotide 59029 and nucleotide 63410 (GenBank Z00044 or NC_001879), respectively (Fig. 1).

Bombardment and regeneration of chloroplast transgenic plants

Plastid transformation was carried out as described in Svab and Maliga (1993) with some modifications. Briefly, one fully expanded, dark-green leaf of tobacco (*Nicotiana tabacum*) 'Petit Havana' grown in sterile culture was placed abaxial side up on RMOP medium (Svab *et al.*, 1990) and the transforming DNA was introduced by the biolistic process using the biolistic device PDS1000/He and 0.6 μm gold particles (Bio-Rad, Hercules, CA). Two days after bombardment, leaf discs (6 mm in diameter each) were cut out from the leaves and transferred to RMOP medium containing either 300 μM 4MI (Acros Organics Morris Plains, NJ, USA) or 300 μM 7MT (Sigma-Aldrich, St Louis, MO, USA). Every other week the plant material was transferred onto new fresh 7MT/4MI-containing RMOP medium and further chopped into small pieces when needed in order to ensure a continuous contact between the leaf material and the selection medium. The 4MI/7MT resistant clones were identified as green shoots 6–12 weeks after bombardment. Leaves from PCR positive shoots were subjected to three additional rounds of regeneration/selection on RMOP-4MI/7MT medium in order to obtain the homoplasmic condition. Resistant shoots from the third round of selection were transferred to the rooting medium with 75 μM of either 4MI or 7MT. Rooted plants were then moved to soil in a greenhouse after gently washing off the agar-solidified rooting medium. Two transformation experiments were performed.

Nucleic acids analysis

Total cellular DNA was extracted using the DNeasy plant kit (Qiagen, Valencia, CA, USA). PCR was carried out to identify the transgene insertion, with *Taq* DNA polymerase (New England Biolabs Inc., Ipswich, MA, USA) for 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1.5 min. The primers used for amplification of a 4.4 kb fragment were *aadAR* (5'-ACCTTAGTGATCTCGCCTTTCACG-3'), located at the end of the *aadA* and *NtrbcL* (5'-TTTGCAGCAGTGGACGTTTTGGATAA-3'), located in the 3' end of the plastid gene *rbcL* (Fig. 1)

For Southern hybridization analysis restriction endonuclease treatment of 5 μg DNA per sample was performed using 5 units μg^{-1} of *ScaI* or *SacI* (New England Biolabs Inc.) enzyme in the manufacturer's buffer, at 37 °C for 5 h. The digested DNA was separated by 0.8% agarose gel electro-

phoresis in 1 \times TAE buffer and then blotted onto a nylon membrane (Hybond-N⁺, GE Healthcare Limited, Little Chalfont, UK) and cross-linked to the membrane by UV. The probes were prepared by PCR using primers designed to amplify either a 815 bp fragment from the ASA2 gene (L40 5'-CTAAAAGCGGGAAGTGGATTCCGC-3' and ASAR 5'-TCTGTACTTCAAATGGGTCAGC-3') or a 758 bp fragment from the *accD* gene (*accD F* 5'-TATAGGGCCG-TTTGTGGTGGTGAA-3' and *accD R* 5'-AATGCAATGTAGGCGTTGGGTTTCG-3') (Fig. 1). The probes were purified with QIAquick PCR purification kit (Qiagen) and then radiolabelled with α -³²P-labelled dCTP (3000 Ci mmol^{-1}) through the random primer method using a Megaprime kit (GE Healthcare Limited). Prehybridization, hybridization, and subsequent washing steps were performed according to the standard protocols (Sambrook *et al.*, 1989). Signals were detected by exposing the blots to X-ray films (Denville Scientific Inc., Metuchen NJ, USA) for 1–5 d at -70 °C.

Total RNA was extracted from young expanded leaves with RNeasy kits (Qiagen) and treated with rDNase I RNase-Free (USB Biochemicals, Cleveland, OH, USA) to eliminate any genomic DNA contamination.

For northern analysis 5 μg of total RNA were separated on a 1% agarose gel in formaldehyde/MOPS buffer, transferred to a nylon membrane and cross-linked to the membrane by UV as described above. The membrane was prehybridized for 2 h at 42 °C in 5 \times SSPE, 5 \times Denhardt's solution, 50% formamide, 0.1% SDS, and 20 mg l^{-1} sheared salmon sperm DNA and then hybridized with the same ASA2 probe used for the Southern analysis at 42 °C overnight. After washing, the membrane signals were detected with X-ray films as described above.

RT-PCR (reverse transcription-polymerase chain reaction) was performed on total RNA treated with rDNase I RNase-Free using the same primer set as for the PCR probe amplification (L40-ASAR). The fragments produced were cloned into a pGEM[®]-T Easy Vector System I (Promega Corporation, Madison, WI, USA) and sequenced. A PCR reaction was performed with the same primers without the reverse transcriptase step to demonstrate the absence of genomic DNA contamination in the samples.

Plant crosses

To obtain seeds to test for the maternal inheritance of the ASA2 gene, the anthers of closed green tobacco flowers, from either WT or the transplastomic plants were removed with tweezers prior to their dehiscence and covered with a plastic bag. At the beginning of colour formation of the petals of the emasculated flowers (1–2 d after emasculation) paternal pollen was collected from either WT or the transplastomic plants, resuspended in sterile distilled water and a 5 μl volume of pollen suspension was transferred onto the top of the stigma of each emasculated flower. Flowers were bagged again and seeds collected after 4–5 weeks. Six randomly chosen lines were used for the plant crosses three selected on 300 μM 7MT (lines 26, 27, 51) and three selected on 300 μM 4MI (A, C, F).

AS assays and Trp measurement

AS enzyme activity was measured in crude protein extracted from 2 g of leaves using the buffer of Bernasconi *et al.* (1994) and desalted using an Econo-Pac 10DG column (Bio-Rad). Protein concentration was determined using a protein dye-binding assay kit (Bio-Rad). AS enzyme activity was measured as the conversion rate of chorismate to anthranilate as described previously (Song *et al.*, 1998). Either 100 mM NH₄Cl or 10 mM glutamine (Gln) was added to the assay mixture to determine α -subunit activity or AS holoenzyme activity, respectively, and Trp at different concentrations to measure feedback inhibition. The total extract of a single leaf of three different plants for each line was analysed.

Free Trp was extracted with 0.1 N HCl from a young expanded leaf from the top of 35 d-old-plants grown in the growth chamber and measured as described by Cho *et al.* (2000).

Results

Selection and regeneration of transgenic plants

Six to twelve weeks after bombardment with pAST-IV, resistant green shoots appeared from the brown tobacco

leaf pieces placed on the selection medium (Fig. 2a, b). Integration of the ASA2 gene into the chloroplast genome was first confirmed by PCR screening of transformants (Fig. 3a) using a primer set (*NtrbcL*–*aadAR*) designed to eliminate lines with nuclear integration of the transgene and escapes (Fig. 1).

In the first experiment, 24 independent resistant clones were obtained from 130 leaves bombarded with vector pAST-IV. Ten clones were recovered from medium containing 7MT and 14 from medium containing 4MI. The PCR screening showed that five (two on 7MT and three on 4MI) out of the 24 putative transformants recovered after the first round of selection were positive, as shown by the presence of the expected 4.4 kb PCR product.

In the second experiment, 32 (15 on 7MT and 17 on 4MI) independent resistant clones were recovered from 140 leaves bombarded. The PCR analysis confirmed six of them to be chloroplast transformants (three on 7MT and three on 4MI). As shown in Fig. 3a no PCR fragment was produced in the WT since primer *aadAR* is located in the coding region of the *aadA* gene. PCR positive lines were subjected to three rounds of additional regeneration/selection with either 7MT or 4MI and then the green shoots were allowed to root and grow into plantlets.

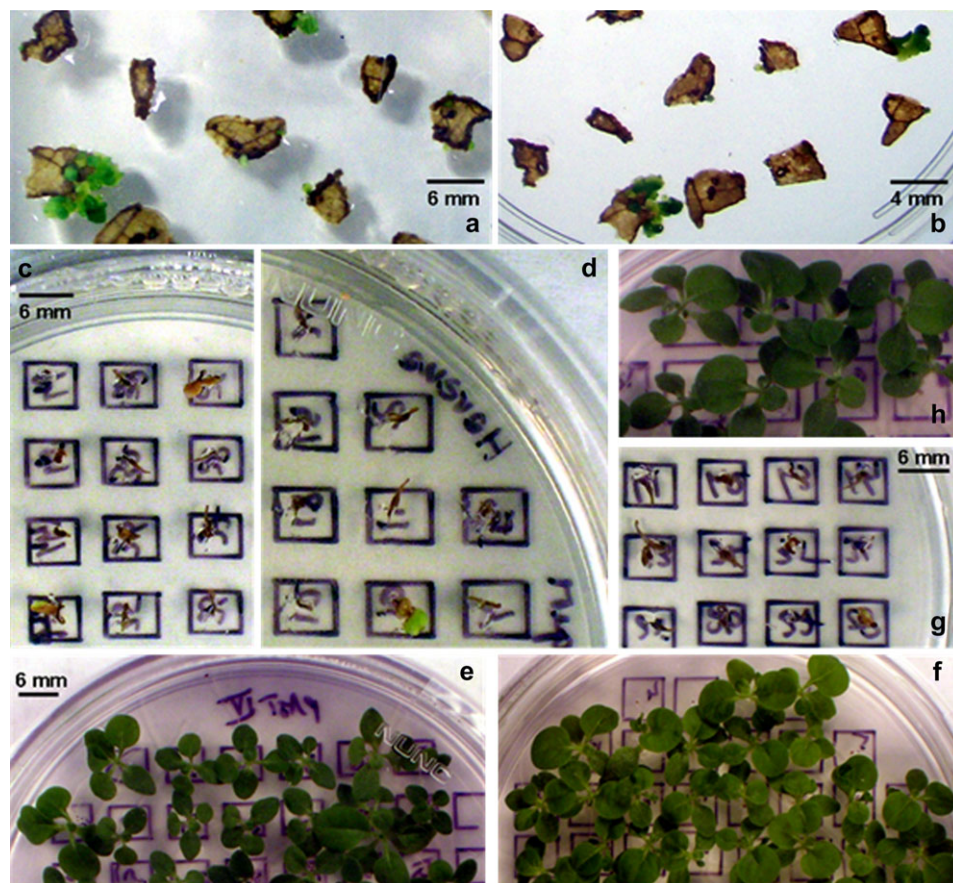


Fig. 2. Bombarded tobacco leaf material on RMOP/7MT 300 μ M (a) or RMOP/4MI 300 μ M (b) with green resistant shoots. Seed germination of WT (*N. tabacum*, Petit Havana) on either 4MI (c) or 7MT (d) 75 μ M. Seedlings from seeds of self-pollinated transplastomic plant 27 on 4MI (e) and plant C on 7MT (f) 75 μ M. Germination of seeds on 7 MT 75 μ M from either WT plant pollinated with pollen from transplastomic plant C (g) or from transplastomic plant C pollinated with pollen from WT (h).

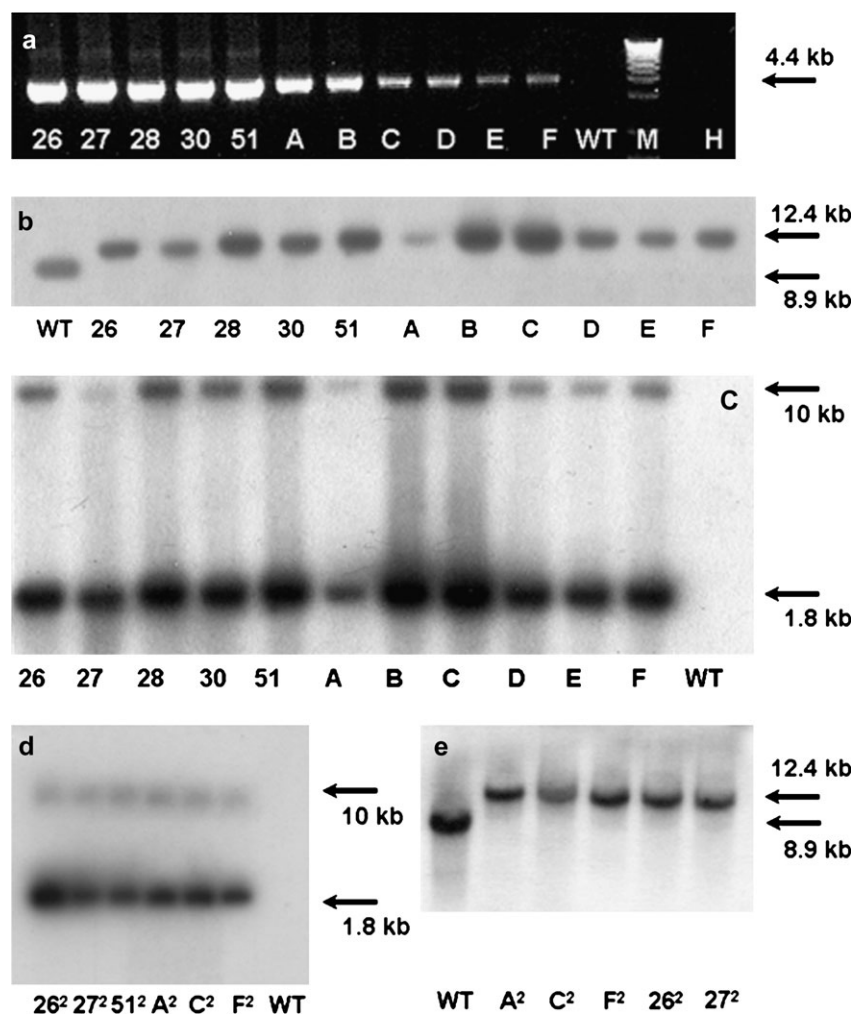


Fig. 3. Plastid genomic analysis of site-specific integration of the ASA2 gene: (a) PCR amplification with primer aadAR (aadA-specific) and primer NtrbcL (plastid site-specific-see Fig. 1); for Southern blot DNA was digested with either *ScaI* and hybridized to the plastid site-specific probe *accD* (b, e) or *SacI* and hybridized to an ASA2 probe (c, d). Lines 26–51 selected on 300 μ M 7MT and lines A–F selected on 300 μ M 4MI. WT, untransformed control; H, blank control/water; M, DNA ladder. Lines 26²–51² F₁ crosses (male non-transgenic \times female transgenic) germinated on 75 μ M 7MT and lines A²–F² F₁ crosses (male non-transgenic \times female transgenic) germinated on 75 μ M 4 MI. WT, untransformed control; H: blank control/water.

Nucleic acids analysis: integration and expression of the transgene

To prove the homoplasmic condition of the transformants after the third round of selection, Southern-blot hybridizations were carried out with either an *accD* gene probe or an ASA2 gene probe. As previously shown in Zhang *et al.* (2001) when hybridized with a 0.7 kb *accD* probe a *ScaI* DNA-digested blot results in an 8933 bp band corresponding to the native plastid *accD-ycf4* region in the WT (GenBank NC_001879; Fig. 1), but generates a 12.4 kb fragment in the pASTIV plants due to the insertion of the Prn-aadA-Prn-ASA2 cassette in the ptDNA (Figs 1, 3b). The *SacI* DNA-digested gel hybridized with a 0.8 kb ASA2 gene probe shows two bands at 1.8 kb and 10 kb (Fig. 3c) for the transformant plants as a result of the two *SacI* sites in the Prn-ASA2 cassette (Fig. 1) and one downstream of *ycf4*. No signal was detected in the WT, even though the tobacco nuclear genome contains a small AS α -subunit gene

family including ASA2 (Zhang *et al.*, 2001), probably due to the relatively low amount of total cellular DNA used. The 1.8 kb band is much stronger because the AS probe covers most of the ASA2 gene (Fig. 1). The same results were obtained with DNA extracted from F₁ crosses (male non-transgenic \times female transgenic) (Fig. 3d, e) confirming the insertion of the ASA2 gene in the ptDNA of the progenies and their homoplastomy.

To determine the expression of the Prn-ASA2 gene in the transformed plants northern-blot hybridization and RT-PCR were performed. The northern analysis showed two transcripts, one of 2.5 kb and one of 7.5 kb (Fig. 4). This result is in accordance with Zhang *et al.* (2001), indicating that the 2.5 kb transcript in the pAST-IV plants ends in the intergenic region between *accD* and *psaI*, whereas the 7.5 kb mRNA is the result of a transcribed operon that includes ASA2 and genes downstream of the *ycf4* gene. ASA2 mRNA could not be detected by northern blot analysis in the leaf of WT plants (Fig. 4) most likely because as

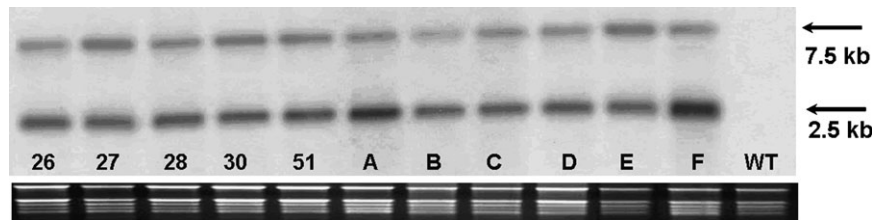


Fig. 4. Northern blot analysis of the total RNA probed with ASA2 probe (see Fig. 1). Plants in lanes 26–51 were selected on 300 μM 7MT and plants in lanes A–F were selected on 300 μM 4MI. WT, untransformed control. The ethidium bromide-stained 26S, 18S, 16S, and 23S rRNAs are shown at the bottom to indicate RNA loading.

previously shown (Song *et al.*, 1998; Zhang *et al.*, 2001) the naturally occurring ASA2 gene is expressed at a very low level in the WT plants.

RT-PCR produced the expected 816 bp PCR fragments from the transgenic plants and the sequence analysis of the fragments confirmed their identity (data not shown).

Maternal inheritance

Germination of seeds from WT and self-pollinated transplastomic plants on rooting medium with either 7MT or 4MI (75 μM) resulted in small brown, WT seedlings that stopped growing after 10–14 d (Fig. 2c, d) whereas for the transformant lines the seedlings were green and normal-looking (Fig. 2e, f). Pollination of the WT plants with pollen from transplastomic plants produced progeny sensitive (Fig. 2g) to the analogues while seeds from transplastomic plants pollinated with the WT pollen were able to germinate on selective medium containing analogues (Fig. 2h) demonstrating the maternal inheritance of the ASA2 gene inserted in the ptDNA.

ASA2 enzyme activity and free Trp measurement

When the AS holoenzyme activity was measured without Trp inhibition the leaf extracts from transformed rooted plants showed activity 4-fold that of the untransformed line (line 27, 96.4 ± 17 pmol min^{-1} mg^{-1} protein; line C, 97.2 ± 37 pmol min^{-1} mg^{-1} protein; WT, 23.05 ± 37 pmol min^{-1} mg^{-1} protein) suggesting that the α -subunits produced by the ASA2 gene in the plastid can functionally couple with the free β -subunits present in the chloroplast, increasing the capacity to convert chorismate into anthranilate.

The AS activity from the transgenic plants showed less sensitivity to Trp inhibition compared to the untransformed tobacco. When the α -subunit activity was measured (Fig. 5a) at 10 μM Trp lines 27 and C still retained about 50% of the activity whereas the WT had about 15% of the activity. Similar results were found for the relative AS holoenzyme activities at 300 μM Trp where the untransformed line is almost completely inhibited while the plastid lines still maintain about 15% of the total activity (Fig. 5b). The estimated K_i values causing 50% inhibition of the AS holoenzyme activity were 2.3, 10.2, and 10.8 μM for WT, line 27, and line C, respectively.

Since the AS enzyme is insensitive to feedback inhibition by Trp, the free Trp levels were measured in three WT

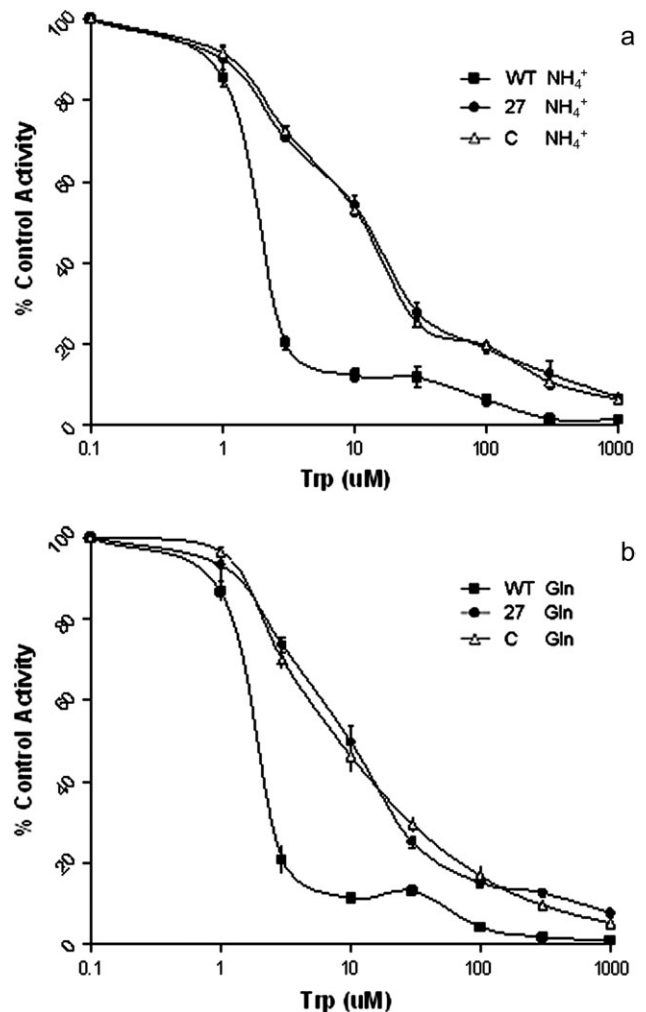


Fig. 5. Trp inhibition of AS enzyme activity in total leaf extracts from wild-type (WT) (filled squares) and transplastomic plants 27 selected on 7MT (filled circles) and C selected on 4MI (open triangles), with 100 mM NH_4Cl (a) (α -subunit activity) or 10 mM Gln (b) (holoenzyme activity) as substrate. The values are means \pm SD of three replicates.

plants, three transgenic lines selected on 7MT (27, 30, 31) and three transgenic lines selected on 4MI (B, C, D). The transplastomic lines showed a higher Trp level averaging 297 ± 5.4 nmol g^{-1} fresh weight for 7MT selected lines and 318 ± 10 nmol g^{-1} fresh weight for 4MI selected lines while the WT had 41 ± 7.2 nmol g^{-1} fresh weight (Fig. 6).

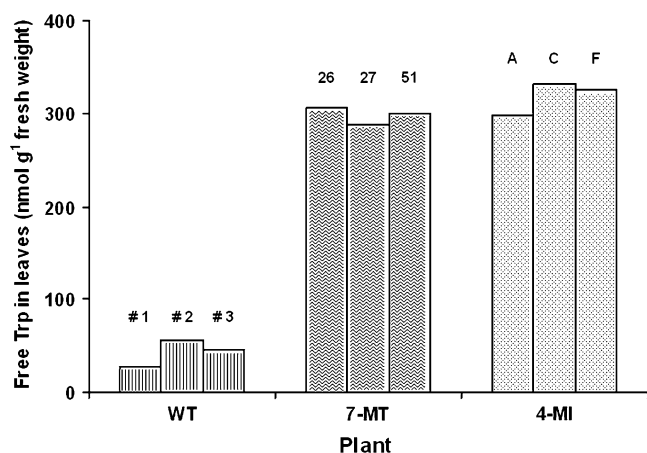


Fig. 6. Free Trp content in leaves of three WT plants and three transplastomic plants selected on either 7MT (26, 27, 51) or 4MI (A, C, F).

Conclusion/discussion

ASA2 has previously been used as a selectable marker gene in nuclear transformation to select the forage legume *Astragalus sinicus* hairy roots with the Trp analogue 5MT as the selection agent (Cho *et al.*, 2004) and tobacco with either the Trp analogue 7MT or the indole analogue 4MI (Barone and Widholm, 2008). Zhang *et al.* (2001) demonstrated that tobacco transplastomic plants expressing the *ASA2* gene and selected on spectinomycin were resistant to 5MT. These reports suggested that a selection system based on a feedback-insensitive AS α -subunit gene as a selectable marker could be a possible tool for chloroplast transformation as well.

Using the *ASA2*/7MT or 4MI selection system, it has been possible to generate transgenic tobacco plants via plastid transformation. The efficiency of transformation is comparable with the one with spectinomycin previously reported in this laboratory with the same vector pAST-IV (Zhang *et al.*, 2001). When the leaf discs were selected for spectinomycin resistance, three individual resistant shoots were obtained from 60 leaves bombarded (Zhang *et al.*, 2001) while under analogue selection five individual resistant lines were obtained from 130 leaves with 7MT and six individual resistant clones were obtained from 140 leaves with 4MI as reported here. The frequency of plastid transformed plants (6) among regenerated shoots (31) was 19.3% for 4MI and 20% for 7MT (5 resistant plants from 25 regenerated shoots) with the rest of the shoots being Trp analogue-resistant mutants/escapes (Widholm, 1972) or nuclear transformants. These values are comparable to the 25.3% reported for spectinomycin (20 resistant plants from 79 regenerated shoots) in Zhang *et al.* (2001).

The transgenic nature of the putative plastid transformants was confirmed by Southern and northern blot analysis. The gene/trait was stably and maternally inherited in the subsequent generation and the chloroplast transgenic lines grew normally, flowered, and set viable seeds showing that the over-expression of *ASA2* gene did not result in any negative effects for the plants.

The plastid transformed plants have about 7-times the normal concentration of free Trp in leaves due to higher level of AS enzyme activity insensitive to Trp-feedback inhibition. Similar increases were found previously in different *ASA2*-expressing species like the forage legume *A. sinicus* (Cho *et al.*, 2000) where a 1.3–5.5-fold increase in free Trp was observed, tobacco (Zhang *et al.*, 2001) with free Trp 10 times higher than in the WT and soybean (Inaba *et al.*, 2007) with a 4–5-fold increase in leaves and embryogenic tissue cultures.

The development of *ASA2* as a chloroplast selective marker is important not only for the biosafety reasons reported above, but also for practical reason due to the scarcity of primary selective markers available. The use of the *ASA2*/7MT or 4MI selection scheme could facilitate the expansion of chloroplast transformation technology to many economically important crops like cereals that are naturally resistant to spectinomycin and for which a specific selection system still has to be established. In addition to the expression of any intended transgene(s) inserted into the plastome, increases in the essential amino acid Trp might (should) also be realized in the transformed plants as a value-added trait. For example, Zhang *et al.* (2001) observed a 27–29% increase in total Trp (free plus protein-bound) levels in mature, dry seeds from the transplastomic plants over-expressing *ASA2*.

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