RESEARCH REPORT

Short title: Plastid Transformation in *Arabidopsis*

Corresponding Author: Pal Maliga, Waksman Institute of Microbiology, Rutgers the State University of New Jersey, Piscataway, New Jersey 08854; email: maliga@waksman.rutgers.edu; phone: 732-763-1333

Title: Efficient Plastid Transformation in *Arabidopsis*

Authors: Qiguo Yu, Kerry Ann Lutz and Pal Maliga

Waksman Institute of Microbiology, Rutgers the State University of New Jersey, Piscataway, New Jersey 08854 (Q.Y., P.M.); Department of Biology, Farmingdale State College, Farmingdale, New York 11735 (K.L.); Department of Plant Biology and Pathology, Rutgers the State University of New Jersey, New Brunswick, New Jersey 08901 (P.M.)

ORCID IDs: 0000-0003-2464-7135 (Q.Y.); 0000-0003-1134-2893 (K.A.L.); 0000-0002-6202-0029 (P.M.)

One sentence summary: 100-fold increased plastid transformation frequency in *ACC2*-defective *Arabidopsis thaliana*. 
Author contribution: P.M. conceived the original research plans; K.A.L. obtained the first transplastomic clones; Q.Y. performed most of the experiments; Q.Y and P.M. analyzed the data; P.M. conceived the project and wrote the article with contributions of all the authors.

Funding Information: This research was supported in part by the NSF Eukaryotic Genetics Program grant MCB-039958 to PM and the Theresa Patnode Santmann Faculty Development Award in Bioscience to KL. Q.Y. is a recipient of a Charles and Joanna Busch Predoctoral Fellowship.

Corresponding author email: maliga@waksman.rutgers.edu
Plastid transformation is routine in tobacco, but 100-fold less frequent in Arabidopsis, preventing its use in plastid biology. A recent study revealed that null mutations in $ACC2$, encoding a plastid-targeted acetyl-CoA-carboxylase, cause hypersensitivity to spectinomycin. We hypothesized that plastid transformation efficiency should increase in the $acc2$ background, because when ACC2 is absent, fatty acid biosynthesis becomes dependent on translation of the plastid-encoded ACC β-Carboxylase subunit. We bombarded $ACC2$-defective Arabidopsis leaves with a vector carrying a selectable spectinomycin resistance ($aadA$) gene and $gfp$, encoding the green fluorescence protein GFP. Spectinomycin resistant clones were identified as green cell clusters on a spectinomycin medium. Plastid transformation was confirmed by GFP accumulation from the second open reading frame of a polycistronic mRNA, that would not be translated in the cytoplasm. We obtained one to two plastid transformation events per bombarded sample in spectinomycin hypersensitive Slavice (Sav-0) and Columbia $acc2$ knockout backgrounds, an approximately 100-fold enhanced plastid transformation frequency. Sav-0 and Columbia are accessions in which plant regeneration is uncharacterized or difficult to obtain. A practical system for Arabidopsis plastid transformation will be obtained by creating an $ACC2$ null background in a regenerable Arabidopsis accession. The recognition that the duplicated ACCase in Arabidopsis is an impediment to plastid transformation provides a rational template to implement plastid transformation in related recalcitrant crops.
INTRODUCTION

Plastids are semi-autonomous plant organelles with thousands of copies of the approximately 155-kb genome localized in 10 to 100 plastids per cell. The plastid genome of higher plants encodes about one hundred genes, the products of which assemble with approximately 3,000 nucleus-encoded proteins to form the plastid transcription and translation machinery and carry out complex metabolic functions, including photosynthesis, and fatty acid and amino acid biosynthesis. Plastid transformation is routine only in tobacco (*Nicotiana tabacum*) (Svab et al., 1990; Svab and Maliga, 1993), but reproducible protocols for plastid transformation have also been described in tomato (Ruf et al., 2001), potato (Valkov et al., 2011), lettuce (Kanamoto et al., 2006; Ruhlman et al., 2010) and soybean (Dufourmantel et al., 2004). Still, the technology is available in only a relatively small number of crops. *Arabidopsis thaliana*, the most widely used model plant is one of the species that is recalcitrant to plastid transformation. In *Arabidopsis*, only 2 transplastomic events were identified in 201 samples (Sikdar et al., 1998), a sample size that would have yielded approximately 200 events in tobacco using the technology available in 1998. Until now the reasons for the low efficiency in Arabidopsis were not understood.

Spectinomycin, the agent for selection of transplastomic events, binds to the 16S ribosomal RNA, blocking translation on the prokaryotic type 70S plastid ribosomes (Wirmer and Westhof, 2006; Wilson, 2014) inhibiting greening and shoot regeneration in tissue culture cells (Svab et al., 1990). When the plastid genome is transformed with the *aadA* gene encoding aminoglycoside-3”-adenylyltransferase (AAD), the AAD protein modifies the antibiotic such that it no longer binds to the 16S rRNA and translation proceeds, enabling greening. Tobacco, when cultured on a spectinomycin medium, bleaches and proliferates at a slow rate due to inhibition of plastid translation. Transplastomic tobacco cells are identified by the ability to green and regenerate shoots in spectinomycin-containing tissue culture medium. In contrast, *Brassica napus*, a close relative of *Arabidopsis thaliana*, bleaches but continues to proliferate as albino shoots on a spectinomycin medium in the absence of chloroplast ribosomes (Zubko and Day, 1998). Two major studies by Parker et al. (2014, 2016) revealed the existence of rare *Arabidopsis* accessions, in which plastids are extremely sensitive to spectinomycin. Seeds of most accessions in the study germinated on spectinomycin and developed into small albino
seedlings or rosettes, including RLD (Reduced number of Long Days, Rschew), the accession used in the 1998 plastid transformation experiment. However, seeds from hypersensitive accessions germinated but did not develop beyond the cotyledonary stage. Genetic analysis

Figure 1. Elimination of ACC2 function makes plastid transformation efficient in Arabidopsis thaliana. A, Heteromeric ACCase (hetACC) localizes in the chloroplast and is encoded by nuclear genes CAC1-A (At5g16390), BCCP1 (At5g15530; BCCP2 (At2g38040), a subunit of Carboxylase (α-CT) and the plastid encoded gene adcD (Atg900500; β subunit of Carboxyltransferase (β-CT)). The homomorphic ACC1 (At1g36170; homACC1) enzyme localizes in the cytoplasm and the ACC2 (At1g36180; homACC2) enzyme is imported into the chloroplast via the TIC/TOC membrane protein complex. If translation of the plastid adcD mRNA is blocked by spectinomycin, the nuclear homomorphic ACC2 gene enables a limited amount of fatty acid biosynthesis, thereby reducing the impact of the absence of heteromeric enzyme in culture, making spectinomycin selection inefficient. B, In hypersensitive acc2 mutants, the absence of the homomeric ACCase makes the cells dependent on plastid translation to produce the heteromeric ACCase enzyme for fatty acid biosynthesis.
revealed that spectinomycin sensitivity of hypersensitive accessions is due to mutations in the
ACC2 nuclear gene. The ACC2 gene produces the homomeric acetyl-CoA-carboxylase
(ACCase) that is imported into plastids, and in tissue culture partially duplicates the function of
heteromeric ACCase, one subunit of which is encoded in the plastid accD gene (Fig. 1A). When
plastid translation is blocked by spectinomycin, no heteromeric ACCase is made, and the
homomeric enzyme enables a limited amount of fatty acid biosynthesis to occur, thereby
reducing the impact of the absence of heteromeric enzyme in culture, making spectinomycin
selection inefficient. In the absence of a functional ACC2 gene, fatty acid biosynthesis is
dependent on the availability of heteromeric ACCase enzyme, the β-carboxylase subunit of
which is translated on plastid ribosomes (Fig. 1B).

We hypothesized that inefficient plastid transformation in our original study was due to
tolerance of Arabidopsis to spectinomycin and that transformation of hypersensitive mutants
defective in ACC2 function should result in efficient recovery of transplastomic clones. We
report here that the efficiency of plastid transformation in the acc2 background is increased
approximately 100-fold and comparable to that of tobacco, confirming our hypothesis.
RESULTS

Plastid Transformation with vector pATV1 and Identification of Transplastomic Events

The plastid transformation vector pATV1 targets insertion upstream of the trnV gene in the inverted repeat region of the plastid genome (Fig. 2A). Vector pATV1 carries a dicistronic operon, in which the first open reading frame (ORF) encodes the aadA spectinomycin resistance gene and the second ORF encodes a green fluorescence protein (GFP) (Fig. 2A). Polycistronic mRNAs are not translated on the eukaryotic-type 80S ribosomes in the cytoplasm, thus accumulation of GFP in chloroplasts in spectinomycin-resistant clones indicates plastid transformation.

Plastid transformation was carried out in the Col-0 (Columbia) accession and the Columbia ACC2 T-DNA insertion line acc2-1 (SALK_148966C), which was shown by Parker et al. (2014) to be hypersensitive to spectinomycin. We also evaluated plastid transformation efficiency in the Sav-0 (Slavice) accession that was the most sensitive to spectinomycin in the study of Parker et al. (2014). The Sav-0 ACC2 gene carries 15 missense mutations, however the hypersensitivity to spectinomycin is thought to be due to one mutation (G135E) that alters a conserved residue immediately preceding the biotin carboxylase domain (Parker et al., 2016).

Plants were grown aseptically on Arabidopsis Revised Medium with 5% sucrose (ARM5 medium) (Fig. 3A); leaves for plastid transformation were harvested from plants grown under aseptic conditions and placed on ARMI media (see Methods). The leaf tissue was bombarded with gold particles coated with vector DNA. After two days, the leaves were stamped with a stack of razor blades, cut into 1 cm² pieces and transferred onto the same medium (ARMI) containing spectinomycin (100 mg/L; Fig. 3B) to facilitate preferential replication of plastids containing transformed ptDNA copies. The ARMI medium induces division of the leaf cells and formation of colorless, embryogenic callus. After 7-10 days of selection on ARMI medium, spectinomycin selection was continued on the ARMIr medium, which induces greening. Since spectinomycin prevents greening of wild-type cells, only spectinomycin-resistant cells formed green calli. Visible green cell clusters appeared within 21 to 40 days on the selective ARMIr medium (Fig. 3C). Illumination of plates with UV light revealed intense fluorescence of GFP in the green calli (Fig. 3D).
In the wild-type Col-0 sample (4 bombarded plates), no transplastomic event was found. We obtained 8 events in 5 bombarded plates using leaf tissue in the acc2-1 mutant background and 4 events in 4 bombarded plates in the Sav-0 accession (Table 1). This transformation...
efficiency is comparable to the transformation efficiency obtained with current protocols in tobacco, 4 to 5 transplastomic events per bombardment (Maliga and Tungsuchat-Huang, 2014).
This is a significant advance, as high frequency plastid transformation in Arabidopsis has been pursued since the publication of the original report in 1998 (Sikdar et al., 1998). Since 2007, 26 plates of RLD and 5 plates of *Landsberg erecta* (Ler) leaf tissue were bombarded; none of which yielded a transplastomic event (Table 1). In contrast, 9 bombardments of leaves with the *acc2* null background yielded 12 transplastomic clones. Even though the technology significantly improved since 1998, no transplastomic clones were obtained until *ACC2* defective leaf tissue was used for bombardments (Table 1), providing overwhelming support for the absence of *ACC2* activity being critical for high frequency plastid transformation in *Arabidopsis thaliana*.

### Confocal Microscopy to Confirm Transplastomic events

GFP is encoded in the second ORF, thus GFP accumulation is expected only if the mRNA is translated in plastids on the prokaryotic type 70S ribosomes known to translate polycistronic mRNAs (Staub and Maliga, 1995). Thus, GFP accumulation was anticipated only if the *gfp* gene is expressed in chloroplasts.

Putative transplastomic lines were identified by green cell cluster formation and were confirmed as transplastomic events by detecting localization of GFP to plastids by confocal microscopy (Fig. 4). Overlay of the GFP and chlorophyll channels indicates that the clones are heteroplastomic, carrying transformed and wild type plastids in the same cells. A good example for mixed plastids is shown in the overlay of GFP and chlorophyll channels in Col-0 *acc2-1* #3 in Fig. 4. The chloroplasts were not well developed in most tissue culture cells. Chlorophyll was detected in only a localized region of plastids in line with thylakoid biogenesis initiating from a localized center (Schottkowski et al., 2012). Good examples are overlays of Col-0 *acc2-1* #5 and Sav-0 #1 in Fig. 4.

The heteroplastomic state detected in the cells of the green clusters was not maintained, and eventually wild-type plastids (ptDNA) disappeared in the callus cells after continued cultivation on selective media. The homoplastomic state is confirmed by uniform accumulation of GFP in the leaves of a Sav-0 #6 plant shown in Fig. 4 and DNA gel blot analyses of calli shown in Fig. 2B.
Regeneration of Transplastomic Sav-0 Plants and Transmission of GFP to Seed Progeny

After bombardment of Col-0 and Sav-0 leaves, selection of transplastomic events was
carried out according to the published RLD protocol (Sikdar et al., 1998). However, when the transplastomic clones were transferred to the RLD shoot-induction medium (ASI-N1B1 medium), the calli did not proliferate. Therefore, we transferred the transplastomic calli to media that were successfully used to regenerate plants from other accessions. We found that the two-step regeneration protocol described for shoot induction in the C24 background (Motte et al., 2013) triggered shoot regeneration in two surviving Sav-0 calli. Calli of Sav-0 transplastomic lines #3 and #6 were briefly (3 days) exposed to callus induction medium containing 0.5 mg/L 2,4-D and 0.05 mg/L kinetin, and then transferred to a shoot regeneration medium containing 0.15 mg/L IAA and 1.6 mg/L phenyl-adenine. Phenyl-adenine is a potent compound for shoot regeneration through inhibition of CYTOKININ OXIDASE/DEHYDROGENASE activity (Motte et al., 2013). Shoots from the calli developed in 45 to 60 days; flowered and formed siliques in sterile culture in 250 ml Erlenmeyer flasks (Fig. 3E). The plants intensely glow when illuminated with UV light, indicating high-level GFP accumulation (Fig. 3F). Confocal microscopy suggests uniform transformation of plastid genomes in the leaves of regenerated Sav-0 #6 plants (Fig. 4) and was confirmed by molecular analyses (Fig. 2B).

The transplastomic shoots were transferred to larger 500 ml Erlenmeyer flasks containing ARM medium for seed set where they continued to grow. It is noteworthy that the shoots did not have any roots or rosette leaves, thus they could be best described as inflorescence cultures. The siliques harvested from the Sav-0 #6 plants were empty, while the Sav-0 #3 shoots produced six seeds. One transplastomic Sav-0 #3 seed germinated on spectinomycin. The cotyledons of this seedling fluoresce under UV light, indicating GFP accumulation (Fig. 3G).

**Molecular Analysis of Transplastomic Arabidopsis Clones**

DNA and RNA gel blot analyses was carried out on the callus and shoots of Sav-0 transplastomic lines #3 and #6. Wild type plastids present in the cells of the green clusters were gradually lost by the time DNA gel blot analyses was carried out, confirming uniform transformation of the plastid genomes in both calli and shoots (Fig. 2B). RNA gel blot analyses indicate the presence of a 2.0-kb dicistronic transcript, detected by both the \( aadA \) and \( gfp \) probes (Fig. 2C).
DISCUSSION
We report here approximately 100-fold enhanced plastid transformation efficiency per bombardment in the *acc2* null background: 8 events in five bombarded samples in the Col-0 *acc2-1* line and 4 events in four bombarded samples in the Sav-0 background. The increase from 1 event per approximately 100 bombardments to 1 event per one bombardment is in part due to technological advances. However, the lack of success with the latest technology in a large number of bombarded samples (Table 1) provides overwhelming evidence that the key to success was the choice of Arabidopsis lines lacking ACC2 activity.

Identification of transplastomic events in the RLD ecotype took 5 to 12 weeks in 1998 (Sikdar et al., 1998). Use of spectinomycin sensitive *acc2* knockout lines and the pATV1 dicistronic operon vector shortened the time period for identification of transplastomic events to 3 to 5 weeks. Use of the *acc2* knockout lines shortened scoring because proliferation of non-transformed cells was efficiently inhibited by spectinomycin, enabling identification of the spectinomycin resistant green cell clusters. Spectinomycin resistance may be due to integration of *aadA* in the plastid genome, integration of *aadA* in the nuclear genome and fortuitous expression from an upstream promoter, or spontaneous mutations in the *rrn16* gene (Svab and Maliga, 1993). GFP, encoded in the second ORF is expressed only in the chloroplasts, enabling rapid identification of transplastomic clones in a small number of heteroplastomic cells by confocal microscopy.

Once transplastomic clones are identified, the next major step is plant regeneration. There is diversity for shoot regeneration potential in Arabidopsis accessions. Columbia is well known for its recalcitrance to shoot regeneration from cultured cells. Therefore, no attempt was made to regenerate shoots from the Col-0 transplastomic callus tissue. There is no information about the tissue culture properties of the Sav-0 accession. Our first attempts at Sav-0 shoot regeneration from the transplastomic clones proved successful, yielding flowering shoots in culture (Fig. 3E). However, the seeds, with one exception, failed to germinate. Seed viability was apparently compromised by somaclonal variation, accumulated genetic changes due to the tissue remaining in culture for close to a year’s time (Bairu et al., 2011).
The first step towards obtaining a system that yields fertile transplastomic Arabidopsis will be obtaining ACC2 null mutations in regenerable accessions. Shoot regeneration protocols have been worked out from root (Marton and Browse, 1991) and leaf explants (Lutz et al., 2015) of the RLD accession; and from protoplasts (Chupeau et al., 2013), leaf explants (Zhao et al., 2014) and inflorescence stem explants (Zhao et al., 2013) of the Wassilewskya (Ws) accession. Thus, RLD and Ws will be our targets for ACC2 mutagenesis.

Plastid Transformation in Arabidopsis Provides Template for Recalcitrant Crops

The recognition that the duplicated ACCase in Arabidopsis is an impediment to plastid transformation provides a rational template to implement plastid transformation in all Arabidopsis accessions, and in crops having a plastid-encoded accD gene and a plastid targeted ACC2 enzyme. The Arabidopsis thaliana ACC2 enzyme has an N-terminal extension compared to ACC1 (Supplemental Fig. 1A). The N-terminal extension is a plastid targeting sequence, as shown by subcellular localization of a GFP fusion protein (Babiychuk et al., 2011). The ACC1 and ACC2 genes are present in most Brassicaceae species, including Arabidopsis lyrata, Camelina sativa, Camelina rubella, Brassica oleracea, Brassica napus and Brassica rapa. The homomeric ACC2 enzyme in these species has an N-terminal extension compared to ACC1 (Supplemental Fig. 1) (Bryant et al., 2011). Thus, a targeted mutation in the ACC2 N-terminal extension should create a spectinomycin hypersensitive variant. Plastid transformation has been achieved in cabbage (Brassica oleracea L. var. capitata L.). Thus, knockout of ACC2 is apparently not necessary to obtain transplastomic events in this crop, at least in the two cultivars tested (Liu et al., 2007; Liu et al., 2008). Plastid transformation in cauliflower (Brassica oleracea var. botrytis) has been obtained at a very low frequency (Nugent et al., 2006). Plastid transformation in oilseed rape (Brassica napus) has also been obtained, but no homoplastomic plants could be obtained (Hou et al., 2003; Cheng et al., 2010). Plastid transformation in Lesquerella fendleri, another oilseed crop in the Brassicaceae, was feasible but inefficient (Skarjinskaia et al., 2003). Deletion of ACC2 in the latter cases is expected to boost plastid transformation efficiency.
CONCLUSION

The experiments reported here establish that plastid transformation frequency is approximately 100-fold higher in ACC2 null mutants. A system to routinely obtain fertile transplastomic Arabidopsis requires ACC2 null mutants in a regenerable accession, in which transplastomic plants can be rapidly obtained.

METHODS
**Tissue Culture Media**

The tissue culture media were adopted from Sikdar *et al.* (1998), originally described by Marton and Browse (Marton and Browse, 1991). The culture media are based on MS salts (Murashige and Skoog, 1962). Arabidopsis Revised Medium (ARM): MS salts, 3% sucrose, 0.8% agar (A7921; Sigma, St. Louis, MO), 200 mg myo-inositol, 0.1 mg biotin (1 mL of 0.1 mg/mL stock) and 1 mL vitamin solution (10 mg vitamin B1, 1 mg vitamin B6, 1 mg nicotinic acid, 1 mg glycine per mL) per liter, pH 5.8. ARM5 medium: ARM medium supplemented with 5% sucrose. ARMI medium: ARM medium containing 3 mg indolacetic acid (IAA), 0.6 mg benzyladenine (BA), 0.15 mg 2,4-dichlorophenoxyacetic acid and 0.3 mg isopentenyladenine (IPA) per liter. ARMIIr medium: ARM medium supplemented with 0.2 mg/L naphthaleneacetic acid and 0.4 mg isopentenyladenine per liter. The stocks of filter sterilized plant hormones and antibiotics (100 mg/L spectinomycin HCl) were added to media cooled to 45°C after autoclaving. Shoot regeneration in the transplastomic Sav-0 clones was obtained on an ARM medium containing 2,4-D (0.5 mg/L), kinetin (0.05 mg/L) and spectinomycin (100 mg/L) (3 days) followed by incubation on an ARM medium containing IAA (0.15 mg/L), Phenyl-Adenine (1.6 mg/L) and spectinomycin (100 mg/L) (Motte et al., 2013). Seed was obtained by growing shoots on MS salt medium containing 3% sucrose, 0.8% agar (A7921; Sigma, St. Louis, MO), pH 5.8.

**Plant Materials and Growth Conditions**

The Sav-0 (CS28725) and Columbia homozygous acc2-1 knockout line (SALK_148966C) seeds were obtained from The Arabidopsis Biological Resource Center, The Ohio State University. The Columbia (Col) seed was obtained from Prof. Juan Dong, Rutgers University. The RLD and Landsberg erecta (Ler) seed was purchased from Lehle Seeds, Round Rock, TX.

For surface sterilization, seeds (25 mg) were treated with 1.7% sodium hypochlorite (5x diluted 8.5% commercial bleach) in a 1.5 ml Eppendorf tube for 15 min. with occasional mixing (Vortex). The bleach was removed by pipetting and washed 3x with sterile distilled water. Seeds were germinated on 50 ml ARM5 medium in deep petri dishes (20 mm high, 10 cm in diameter). The plates were illuminated for 8 hours using cool-white fluorescent tubes (2,000 lux). The seeds
germinated after 10-15 days of incubation at 24°C. To grow plants with larger leaves, seedlings were individually transferred to ARM5 plates (4 plants per deep petri dish). The plates were illuminated for 8 hours with cool-white fluorescent bulbs (2000 lux) and incubated at 21°C during the day and 18°C during night. 1 to 2 cm long, dark green leaves were harvested for bombardment after incubation for an additional 5 to 6 weeks.

**Plastid Transformation and Selection of Transplastomic Lines**

The plastid transformation vector pATV1 reported here targets insertion in the inverted repeat region of the plastid genome upstream of the trnV gene (Fig. 2). The DNA sequence was deposited in GenBank under accession no. MF461355. The pAAK176 and pTT626 plastid transformation vectors share the plastid-targeting region with vector pATV1. The pAAK176 vector carries the Prrn:LrbcL:aadA:TpsbA marker gene present in vector pHK34 (Kuroda and Maliga, 2001). The aadA gene is between two loxP sites which facilitate the excision of the marker gene, leaving behind a loxP target site (Lutz et al., 2004). The pTT626 plastid transformation vector encodes aadA-gfp fusion protein (Khan and Maliga, 1999) in PrrnLcry/TpsbA cassette (Chakrabarti et al., 2006).

Plastid transformation in Arabidopsis was carried out using our 1998 protocol, as shown in Fig. 3 (Sikdar et al., 1998). The leaves (each 10 to 20 mm) were harvested from aseptically grown plants and covered the surface of agar-solidified ARMI medium in a 10 cm petri dish. We used 100 to 120 leaves to cover the surface of the plate. The leaves were cultured for 4 days on ARMI medium, then bombarded with pATV1 vector DNA. Transforming DNA was coated on the surface of microscopic (0.6 μm) gold particles, then introduced into chloroplasts by the biolistic process (1,100 psi) using a helium-driven PDS1000/He biolistic gun equipped with the Hepta-adaptor (Lutz et al., 2011). The plates were placed on the shelf at the lowest position for bombardment.

Following bombardment, the leaves were incubated for two additional days on ARMI medium. After this time period, the leaves were stamped with a stack of 10 razor blades to create parallel incisions 1 mm apart. The stamped leaves were cut into smaller (1 cm²) pieces and
transferred onto the same medium (ARMI) containing 100 mg/L spectinomycin and incubated at 28°C illuminated for 16 hours with fluorescent tubes (CXL F025/741). After 8 to 10 days, the leaf strips were transferred onto selective ARMIr medium containing 100 mg/L spectinomycin for the selection of spectinomycin resistant clones. The leaf strips were transferred to a fresh selective ARMIr medium every two weeks until putative transplastomic clones were identified as resistant green calli.

**Confocal Microscopy to Detect GFP in Plastids**

Subcellular localization of GFP fluorescence was determined by a Leica TCS SP5II confocal microscope. To detect GFP and chlorophyll fluorescence, excitation wavelengths were at 488 nm and 568 nm, and the detection filters were set to 500-530 nm and 650-700 nm, respectively.

**DNA and RNA Gel Blot Analyses**

Total leaf DNA was prepared by the cetyltrimethylammonium bromide (CTAB) protocol (Tungsuchat-Huang and Maliga, 2012). DNA gel blot analyses was carried out as described (Svab and Maliga, 1993). Total cellular DNA was digested with the EcoRI restriction enzyme. The DNA probe was the *ApaI-SphI* ptDNA fragment encoding the plastid *rrn16* gene (Fig. 2).

Total cellular RNA was isolated from leaves frozen in liquid nitrogen using TRIzol (Ambion/Life technologies), following the manufacturer’s protocol. RNA gel blot analyses was carried out as described (Kuroda and Maliga, 2001). The probes were: *aadA*, 0.8-kb *NcoI-XbaI* fragment isolated from plasmid pHCl (Carrer et al., 1990); *gfp*, fragment amplified from *gfp* coding region using primers *gfp*-forward p1(5’-TTTCTGTCAGTGGAGAGGGTG-3’) and *gfp*-reverse p2 (5’-CCCAGCAGCTGTACAAACT-3’; Fig. 2).

**Alignment of Homomeric ACCases**

Alignment of homomeric ACCases in the Brassicaceae family was carried out with the MultAlin software (Corpet, 1988).
Accession Numbers

DNA sequence of the pATV1 Arabidopsis plastid transformation vector is deposited in GenBank under accession number MF461355.

ACKNOWLEDGEMENTS

We thank Arun K. Azhagiri and Tarini Tungsuchat Huang for plastid transformation vectors pAAK176 and pTT626, respectively.

Supplemental Data

Supplemental Figure: Alignment of homomeric ACCases in the Brassicaceae family.
Table 1. Identification of transplastomic events in Arabidopsis

Au, gold particles; Hepta, using the Biolistic gun Hepta adaptor instead of a single flying disk; Tu, tungsten particles.

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<th>Accession</th>
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Figure 1. Elimination of ACC2 function makes plastid transformation efficient in *Arabidopsis thaliana*. A, Heteromeric ACCase (hetACC) localizes in the chloroplast and is encoded by nuclear genes *CAC1-A* (At5g16390; Biotin Carboxyl Carrier Protein 1 (BCCP-1)), *CAC1-B* (At5g15530; Biotin Carboxyl Carrier Protein 2 (BCCP-2)) (not depicted), *CAC2* (At5g35360; Biotin Carboxylase (BC)), *CAC3* (At2g38040, α subunit of Carboxyltransferase (α-CT) and the plastid encoded gene *accD* (AtCg00500; β subunit of Carboxyltransferase (β-CT)). The homomeric ACC1 (At1g36160; homACCase) enzyme localizes in the cytoplasm and the ACC2 (At1g36180; homACCase) enzyme is imported into the chloroplast via the TIC/TOC membrane protein complex. If translation of the plastid *accD* mRNA is blocked by spectinomycin, the nuclear homomeric *ACC2* gene enables a limited amount of fatty acid biosynthesis, thereby reducing the impact of the absence of heteromeric enzyme in culture, making spectinomycin selection inefficient. B, In hypersensitive acc2 mutants, the absence of the homomeric ACCase makes the cells dependent on plastid translation to produce the heteromeric ACCase enzyme for fatty acid biosynthesis.

Figure 2. Molecular characterization of the Sav-0 transplastomic clones. A, Map of the plastid genome with the integrated *aadA-gfp* dicistronic operon. The *NruI-XbaI* region is contained in the plastid transformation vector pATV1. P and T mark the position of PrnrLatpB promoter and TpsbA terminator in the dicistronic vector. The black box at the *aadA* N-terminus marks the *atpB* Downstream Box sequence (Kuroda and Maliga, 2001). The ribosome entry site is marked by black semi-ovals. Positions of the *rrn16* and *trnV* plastid genes and relevant restriction enzyme sites are marked. Thick black and red lines indicate probes used for DNA and RNA gel blot analysis, respectively. B, DNA gel blot using the *rrn16* probe (Fig. 2A) indicates that the transplastomic Sav-0 calli and leaves are homoplastomic, carrying only the 4.7-kb *EcoRI* fragment and lacking the 2.7-kb wild type fragment. C, RNA gel blot analyses using both the *aadA* and *gfp* probes (Fig. 2A) recognize the same 2 kb dicistronic mRNA.

Figure 3. Identification of Arabidopsis transplastomic clones. A, Sterile Sav-0 plants grown in Petri dishes (diameter 10 cm) for six weeks. B, Two days after bombardment the Sav-0 leaves
are incised and transferred to selective spectinomycin (100 mg/L) medium. C, Sav-0 leaves on selective medium one month after bombardment. Note scanty callus formation and green cell cluster (arrow). D, Culture shown in Fig. 3C, illuminated with UV light. Note green fluorescence indicating GFP accumulation in green cell cluster. E, Sav-0 plant regenerated from a transplastomic clone #6 in sterile culture. F, Culture shown in Fig. 3E, illuminated with UV light. G, Sav-0#3 seed progeny illuminated with UV light. The bar is 1 mm.

**Figure 4.** The green fluorescent protein (GFP) accumulates in chloroplasts. Shown are confocal images collected in the GFP, chlorophyll, and merged channels on a Leica TCS SP5II confocal microscope. Excitation wavelengths were at 488 nm and 568 nm, detection at 500-530 nm and 650-700 nm, respectively. Note absence of GFP and chlorophyll in the wild-type Col-0 callus cells and mixed GFP expressing transgenic and wild type plastids in the Col-0-acc2-1#1 and Sav-0 #6 lines. Note the absence of wild type plastids in the leaves of Sav-O#6 plants. Yellow color in the merged images indicates co-localization of GFP to chlorophyll in plastids. Bar represents 10 μm. Note that cells in the small green cell clusters are heteroplastomic. The only exception are cells in Sav-0#6 leaves, which are homoplastomic due to prolonged selection in tissue culture.

**Supplemental Fig. 1.** Alignment of homomeric ACCases in the Brassicaceae family. A, Alignment of 200 the N-terminal amino acids of *Arabidopsis thaliana* ACC1 (At1g36160) and ACC2 (At1g36180) genes. B, Alignment of 200 the N-terminal amino acids of *Arabidopsis thaliana* ACC1: At1g36160; *Arabidopsis lyrata* ACC1: XM_002891166.1; *Camelina sativa* ACC1-1: LOC104777496; *Camelina sativa* ACC1-2: LOC104743830; *Capsella rubella* ACC1: LOC106413885; *Brassica oleracea* ACC1: LOC106311006; *Brassica napus* ACC1-1: LOC106413885; *Brassica napus* ACC1-2: LOC106418889; *Brassica rapa* ACC1: LOC103833578. C, Alignment of 300 the N-terminal amino acids of *Arabidopsis thaliana* ACC2: At1g36180; *Arabidopsis lyrata* ACC2: XM_002891167.1; *Camelina sativa* ACC2-1: LOC104777495; *Camelina sativa* ACC2-2: LOC104742086; *Capsella rubella* ACC2: CARUB_v10011872mg; *Brassica oleracea* ACC2: LOC106301042; *Brassica napus* ACC2-1: Y10302; *Brassica napus* ACC2-2: X77576; *Brassica rapa* ACC2: LOC103871500.

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