High-efficiency generation of fertile transplastomic Arabidopsis plants

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The development of technologies for the stable genetic transformation of plastid (chloroplast) genomes has been a boon to both basic and applied research. However, extension of the transplastomic technology to major crops and model plants has proven extremely challenging, and the species range of plastid transformation is still very much limited in that most species currently remain recalcitrant to plastid genome engineering. Here, we report an efficient plastid transformation technology for the model plant Arabidopsis thaliana that relies on root-derived microcalli as a source tissue for biolistic transformation. The method produces fertile transplastomic plants at high frequency when combined with a clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9)-generated knockout allele of a nuclear locus that enhances sensitivity to the selection agent used for isolation of transplastomic events. Our work makes the model organism of plant biology amenable to routine engineering of the plastid genome, facilitates the combination of plastid engineering with the power of Arabidopsis nuclear genetics, and informs the future development of plastid transformation protocols for other recalcitrant species.

Stable transformation of chloroplast genomes in the unicellular green alga Chlamydomonas reinhardtii and the seed plant tobacco Nicotiana tabacum was achieved more than 25 years ago. Since then, the technology has proven highly valuable in both basic research and biotechnology. It paved the way to the study of all steps in plastid gene expression and their regulation in vivo, facilitated systematic investigation of the functions of chloroplast genes and open reading frames by reverse genetics, and enabled experimental reconstruction of key processes in genome evolution. In addition, transplastomic technology opened up new applications in metabolic engineering, molecular farming and resistance engineering.

Given the transformative nature of this technology in so many research areas, enormous efforts have been made in both academic and commercial sectors to extend the chloroplast transformation technology to other algae and plants—most importantly, model species and agriculturally important crops. However, extending the species range of the technology has proven extremely difficult, and over the past 25 years, only very few species could be added to the list of transformable plants. These include a few solanaceous species, lettuce and poplar. What all these plants have in common is that they display favourable properties in vitro culture and are relatively easy to regenerate. Thus, while biolistic transformation provides a universal, species-independent method for the introduction of foreign DNA into plastids, the efficient selection of transplastomic events and their regeneration into fertile plants represents the major obstacle to expansion of the species range of the transplastomic technology.

For the above reasons, plastid transformation has also proven to be a serious challenge in the model system of plant biology, Arabidopsis thaliana—a member of the mustard family (Brassicaceae). While the production of plastid-transformed Arabidopsis cells by biolistic bombardment of leaves was accomplished as early as the year 1998, the regenerated plants were male and female sterile and thus could not be maintained. Recent work has made the generation of transplastomic Arabidopsis cells more efficient, but has not solved the fertility problem. This is unsurprising, given that the nuclei of Arabidopsis leaf cells are highly polyploid, with the average ploidy level in mature rosette leaves being 13C (ref. 25). It is for this reason that all methods that have been routinely used for Arabidopsis nuclear transformation rely on non-leafy source tissues (for example, agroinfection of roots, vacuum infiltration of flowers and floral dip).

Here, we report the development of an efficient plastid transformation protocol for Arabidopsis. The system uses microcallus material derived from roots as source tissue and, when combined with the knockout of a nuclear locus that determines sensitivity to the selection agent spectinomycin, produces fertile transplastomic plants at high frequency. Our work makes Arabidopsis amenable to routine engineering of the plastid genome, opens up the possibility to combine the power of Arabidopsis nuclear genetics with chloroplast genome manipulations, and will probably enable new synthetic biology applications in Arabidopsis chloroplasts.

Results

A root-based tissue culture and selection system for Arabidopsis plastid transformation. We reasoned that the problem with obtaining fertile transplastomic Arabidopsis plants can only be overcome by the use of a source tissue for transformation that readily regenerates and is largely diploid. Regeneration from root tissue initiates from the pericycle—a one-layer cylinder of cells separating the endodermis from the stele. The pericycle cells are meristematic, largely diploid and, in intact plants, play a key role in the initiation of lateral roots. Protocols for nuclear transformation of Arabidopsis root tissue had been developed before vacuum infiltration and floral dip obviated the need for tissue culture in Arabidopsis nuclear transgenesis 25 years ago.

To optimize root regeneration for Arabidopsis chloroplast transformation, we chose A. thaliana C24—a standard ecotype that is widely used (for example, in research on abiotic and biotic
harvested from a lawn of young seedlings raised on synthetic medium as a starting material (Supplementary Fig. 1). Alterations in the hormone composition (that is, reduction of the concentration of 2-isopentenyladenine to 2 mg l\(^{-1}\) and inclusion of the growth-promoting peptide hormone phytosulfokine; see Methods) improved the general responsiveness of the root-derived microcallus tissue that was used as source material for transformation experiments to shoot induction and plant regeneration (Supplementary Fig. 2). Nuclear transformation experiments with standard vectors containing the kanamycin resistance gene \(nptII\) as a selectable marker were conducted to optimize the parameters of the biolistic bombardment and selection and regeneration conditions (see Methods and Supplementary Fig. 3). The optimized system produced nuclear transgenic lines at high frequency (on average, five to ten transgenic lines per bombarded sample; Supplementary Fig. 3).

Chimeric \(aadA\) genes that confer resistance to spectinomycin represent the standard selectable marker gene for transformation of the chloroplast genome\(^{30-31}\). Antibiotic sensitivity tests revealed that \(Arabidopsis\) cells are much more sensitive to spectinomycin than tobacco cells, and they bleached out completely when exposed to concentrations as low as 5 \(\mu\)g ml\(^{-1}\) spectinomycin. However, consistent with previous findings\(^{30-34}\), we also noted that the bleached cells displayed a remarkable capacity to continue to proliferate in the presence of spectinomycin (Supplementary Fig. 4). Spectinomycin concentrations of 10–50 \(\mu\)g ml\(^{-1}\) were found to suppress the growth of untransformed callus reasonably well and were therefore used for our initial sets of plastid transformation experiments. However, full suppression of background growth turned out not to be possible (Supplementary Fig. 4).

Vector development and \(Arabidopsis\) chloroplast transformation. Based on previously developed vectors for plastid transformation of solanaceous species\(^{32}\), a set of vectors for transformation of the chloroplast genome of \(Arabidopsis\) was constructed. To this end, the homologous region from the \(Arabidopsis\) plastid genome was cloned (Fig. 2a). Initially, both chimeric \(aadA\) cassettes and \(aphA-6\) cassettes were tested as selectable markers (Table 1). The \(aadA\) gene confers resistance to spectinomycin\(^{32}\), whereas \(aphA-6\) confers resistance to kanamycin\(^{32}\). Since plastid gene expression is generally much less active in non-green tissues than photosynthetically active leaf tissues\(^{36-38}\), we also constructed marker cassettes with expression signals that had been optimized for high-level expression in root plastids\(^{39}\). In these vectors (Table 1 and Fig. 2a), the marker gene is driven by the promoter and 5′ untranslated region (UTR) from the plastid \(clpP\) gene, which is further enhanced by inclusion of the strong Shine–Dalgarno sequence from \(Escherichia coli\) (ref. 39). To further optimize marker expression, we also constructed an \(aadA\) expression cassette with a synthetic codon-optimized \(aadA\) gene. In this cassette, the \(rrnB\) leader sequence (T7 promoter) from \(Escherichia coli\) was used as the 3′ UTR (Fig. 2a). This sequence was reported to confer higher transcript stability than plastid 3′ UTRs (ref. 40). As visual reporters of transcript expression, chimeric \(yfp\) and \(gfp\) cassettes were additionally incorporated into some vectors (Fig. 2a and Table 1).

Large-scale plastid transformation experiments were conducted using the biolistic method and our optimized root-based regeneration system combined with either spectinomycin or kanamycin selection. Testing seven different vectors and performing more than 1,350 biolistic bombardments (Table 1), a single transplastomic event was obtained (Fig. 2b and Supplementary Fig. 4). The line was obtained with vector pCH8, which carries the spectinomycin resistance gene \(aadA\) driven by the \(clpP\)-derived expression signals optimized for expression in non-green tissues\(^{39}\). This promoter-marker gene combination was therefore used in all of the subsequent transformation experiments.
The transplastomic line At-CH8-1 was characterized by Southern blot analysis (Fig. 2), which confirmed its transplastomic status and revealed homoplasm (that is, the absence of residual copies of the wild-type plastid genome). The line could be readily regenerated into fertile plants that produced abundant seeds (Fig. 1b), suggesting that our root-based tissue culture and regeneration protocol may solve the plant sterility problem in Arabidopsis chloroplast transformation.

**Generation of acc2 knockout alleles by clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9) editing.** We reasoned that the very low efficiency at which transplastomic events could be obtained was due to the inefficiency of selection (see above) and the rampant growth of untransformed callus material, which probably prevented the successful outgrowth of transplastomic cell lines (Fig. 2b and Supplementary Fig. 4). Recent work has revealed that the sensitivity of Arabidopsis to inhibition of chloroplast translation is largely determined by the nuclear ACC2 locus\(^{26,27}\). ACC2 encodes an alternative acetyl-CoA carboxylase (ACCCase) that, on inhibition of expression of the plastid-encoded accD gene, sustains de novo fatty acid biosynthesis, thus promoting cell proliferation in the presence of chloroplast translational inhibitors. Arabidopsis ecotypes that harbour ACC2 alleles with low activity (or null alleles) display enhanced sensitivity to spectinomycin\(^{26,27}\), but do not show favourable properties in tissue culture. Therefore, although they can produce transplastomic cell lines at higher frequencies, they are not helpful for the generation of fertile transplastomic plants\(^{26,27}\).

To improve the recovery rate of transplastomic events in our plastid transformation system, we set out to generate acc2 null alleles in the *A. thaliana* ecotype C24 with the help of the CRISPR–Cas9 system for genome editing\(^{28}\). Several putative knockout alleles were obtained, two of which were characterized in detail (Supplementary Fig. 5). While one allele turned out to be a deletion allele, the other showed a fragment inversion at the Cas9 cleavage sites in the ACC2 locus (Supplementary Fig. 5b). Tests for inhibition of seedling growth by spectinomycin revealed complete growth arrest in the two genome-edited mutants (while the C24 wild type continued to proliferate), strongly suggesting that both alleles represent functional acc2 knockout alleles (Supplementary Fig. 5c).

To produce suitable (non-transgenic) recipient lines for plastid transformation, the *cas9* gene was segregated out, and homozygous acc2 knockout mutants were produced for both alleles. These transgene-free lines (subsequently referred to as At-Δacc2 recipient lines) were used for all subsequent plastid transformation experiments. Consistent with previous reports\(^{26,27}\), our acc2 knockout mutants showed no visible phenotype, and their growth and development was indistinguishable from the wild type under all conditions tested. This is unsurprising, given the redundancy of the ACC2 enzyme and the predominant reliance of de novo fatty acid biosynthesis on the heteromeric acetyl-CoA carboxylase in the plastid.

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**Fig. 2 | Construction of plastid transformation vectors and selection of transplastomic Arabidopsis plants.** a. Physical map of the targeting region in the plastid genome (ptDNA) of Arabidopsis and plastid transformation vectors. Genes above the line are transcribed from left to right, while genes below the line are transcribed from the opposite strand of the ptDNA. The transgenes are inserted into the intergenic spacer between the trn*M and trn*G genes within a cloned ptDNA fragment\(^{28}\) (transformation vector). The locations of two BglII restriction sites that were used for Southern blot analysis, and the binding site of the hybridization probe, are also indicated. The sizes of the transgene cassettes in the three vectors are given in kilobases (kb). a-CH8-3 At-CH8-1/uni2206 At-CH8-1/uni2206 At-CH8-1/uni2206 a-JF1153-1 At-JF1153-2 At-JF1153-2 At-JF1153-2 a-JF1151-1 At-JF1151-1 At-JF1151-1 At-JF1151-1

b. Selection of a transplastomic line (white arrowhead) following bombardment of wild-type tissue with vector pCH8. These transformation experiments were repeated independently 507 times (see Table 1) and resulted in similar background growth of the bombarded calli. c. Selection of a transplastomic line (white arrowhead) after bombardment of *acc2* knockout tissue (At-Δacc2) with vector pCH8. Note the much more efficient suppression of background callus growth from the *acc2* knockout tissue. For additional images, see Supplementary Figs. 4 and 6. The transformation experiments with the At-Δacc2 recipient line and vector pCH8 were repeated independently 98 times with similar results. d. Southern blot analysis of transplastomic Arabidopsis lines. Total DNA extracted from regenerated plants growing under aseptic conditions was digested with BglII, separated by agarose gel electrophoresis and hybridized to a radiolabelled probe (see a). The sizes of hybridizing fragments are indicated in kb to the right. These experiments were repeated independently three times with similar results.
### Table 1 | Summary of transformation experiments conducted in A. thaliana

| Vector     | Transgene cassettes | Selection | Recipient | Bombarded plates | Resistant lines | Resistant lines analysed | Confirmed transplastic lines | Spontaneous mutants | Lines grown to maturity | Lines with seeds obtained |
|------------|---------------------|-----------|-----------|------------------|-----------------|------------------------|-----------------------------|---------------------|--------------------------|--------------------------|
| pSTS5      | NPrm:zbdA::aadA::NtTpsbA | Spectinomycin | WT | 60 | 0 | 0 | 0 | 0 |
| pSTS7      | NPrm:G10:aadA::NtTpsbA | Spectinomycin | WT | 155 | 0 | 0 | 0 | 0 |
| pSTS10     | NPrm:zbdA::aadA::NtTpsbA CrPrmG10:gap::CrTatpA | Spectinomycin | WT | 92 | 0 | 0 | 0 | 0 |
| pSTS12     | NPrm:G10:aadA::NtTpsbA CrPrmG10:gap::CrTatpA | Spectinomycin | WT | 90 | 1 | 1 | 0 | 1 |
| pSTS13     | NPrm:G10::aphA-6::NtTpsbA CrPrmG10:gap::CrTatpA | Kanamycin | WT | 192 | 0 | 0 | 0 | 0 |
| pCH7       | ZmPulpPG10::aphA-6::NtTpsb16 | Kanamycin | WT | 259 | 0 | 0 | 0 | 0 |
| pCH8       | ZmPulpPG10::aadA::NtTpsb16 | Spectinomycin | WT | 507 | 1 | 1 | 1 | 0 | 1 | 1 |
| pCH8       | ZmPulpPG10::aadA::NtTpsb16 | Spectinomycin | Δacc2 | 98 | 43 | 30 | 29 | 1 | 8 | 6 |
| pJF1153    | ZmPulpPG10::syn::aadA::EcTrm8 | Spectinomycin | Δacc2 | 141 | 43 | 34 | 30 | 4 | 14 | 12 |
| pJF1151    | ZmPulpPG10::syn::aadA::EcTrm8 CrPsbA::yfp::CrTatp8 | Spectinomycin | Δacc2 | 18 | 6 | 5 | 5 | 0 | 3 | 3 |

Cr, C. reinhardtii; Ec, E. coli; G10, gene10 leader from bacteriophage T7; Nt, N. tabacum; P, promoter; T, 3' UTR; WT, wild type; Zm, Z. mays.

Greatly enhanced plastid transformation efficiency with acc2 recipient lines. Next, we used root-derived microcalli from the At-Δacc2 line as a source material for large-scale plastid transformation experiments (Table 1). Bombardments were conducted with three vectors, all carrying the aadA marker that is controlled by the expression elements optimized for root tissue46: pCH8 harbouring the non-codon-optimized aadA; pJF1153 containing the synthetic, codon-optimized aadA; and pJF1151 carrying a yfp gene in addition to the synthetic aadA (Fig. 2a and Table 1). All three vectors produced large numbers of spectinomycin-resistant lines (At-Δa-CH8, At-Δa-JF1151 and At-Δa-JF1153 lines; Table 1)—on average, one line per three shots. With the exception of a few lines probably representing spontaneous spectinomycin-resistant mutants46, the transplastomic status of all lines analysed could be confirmed by PCR assays and/or DNA gel blot analyses (Fig. 2d and Table 1). When compared with transformation experiments with the wild type, the growth of untransformed callus tissue was much more strongly suppressed by spectinomycin in the At-Δacc2 tissue (Fig. 2h,c and Supplementary Figs. 4 and 6), indicating that the ACC2 gene knockout is indeed responsible for the greatly improved transformation efficiency (Table 1).

It is important to note that the transformation efficiency (of approximately one transplastomic event per three shots; Table 1) represents the most conservative estimate and, in reality, is likely to be significantly higher. This is because multiple resistant lines appearing from one bombarded sample were counted as a single transplastomic event. This conservative approach was taken because, due to the callus transfer to fresh plates involved in the protocol (Supplementary Fig. 6h,i), it cannot be excluded with certainty that, occasionally, a transplastomic event was split into two calli. Given that, if it occurs at all, this should be a rather rare event and we frequently detected more than one transplastomic callus per plate, we believe that we significantly underestimate the transformation frequency.

YFP reporter expression and maternal transgene inheritance. Transplastomic calli could be readily regenerated into plantlets (Supplementary Figs. 6 and 7). Interestingly, all regenerated plants examined were homoplasmic (Figs. 2d and 3), thus obviating the need to conduct the additional cycles of regeneration and selection that normally need to be performed in plastid transformation to eliminate residual copies of the (highly polyploid) wild-type plastid genome. This observation suggests that, in our tissue culture and selection system, transplastomic lines attain the homoplasmic state very quickly, presumably due to the high selection pressure exerted by the antibiotic.

The transplastomic lines flowered readily and produced abundant numbers of seeds (Fig. 1b and Supplementary Figs. 7 and 8). To ultimately confirm homoplasy, transgene inheritance assays were performed. To this end, the transplastomic lines were selfed and reciprocally crossed to wild-type plants. Germination of seeds on medium with spectinomycin revealed a uniform population of resistant seedlings in the progeny of selfed plants and crosses with the transplastomic plant as the maternal parent (Fig. 3). In contrast, crosses with the transplastomic plant as the paternal parent (and the wild type as the maternal parent) yielded a uniform population of antibiotic-sensitive seedlings, in line with the maternal inheritance of the plastid genome47–49.

The successful production of transplastomic At-Δa-JF1151 plants that additionally carry a yfp cassette enabled us to: (1) also test for uniparental inheritance of the fluorescent reporter; and (2) analyse subcellular localization of the YFP fluorescence (Fig. 4). The presence of bright YFP fluorescence in chloroplasts (Fig. 4b) and the strong fluorescence of the entire At-Δa-JF1151 seedlings (Fig. 4a) confirmed high-level expression of the reporter protein and its confinement to the chloroplast compartment.

Finally, we performed immunoblot analyses to determine the YFP expression level in the transplastomic lines. Using a dilution series of recombinant YFP as standard, we estimated YFP to accumulate to approximately 1–2% of the total soluble protein (Fig. 5).

**Discussion**

Our work reported here has overcome the main obstacle preventing the extension of the transplastomic technology to the model plant A. thaliana. Given that the introduction of foreign DNA into chloroplasts by biolistic transformation solely relies on physical principles and therefore occurs in a largely species-independent manner, the development of efficient protocols for the selection and regeneration of fertile transplastomic lines is key to the establishment of plastid transformation in any new species. The inefficiency of regeneration from leaves and the high incidence of (male and female) infertility of the regenerated plants has hampered the
development of a transplastomic technology for Arabidopsis, even though transplastomic cells could be readily produced\textsuperscript{22,23}. As noted previously\textsuperscript{23}, the major “challenge is therefore to identify procedures and ecotypes that facilitate this conversion of transplastomic callus of Arabidopsis into stable and heritable plant material”.

The keys to our success in the establishment of a workable transplastomic protocol for Arabidopsis were: (1) the use of a root-based selection and regeneration system that does not suffer from somatic endopolyploidization, and therefore facilitates the efficient regeneration of fertile plants; and (2) the knockout of the nuclear ACC2 locus that had been shown previously to determine the tolerance of Arabidopsis cells to spectinomycin\textsuperscript{24,25}, thus facilitating the selection of spectinomycin-resistant cells\textsuperscript{7}. Although we were also able to obtain fertile transplastomic Arabidopsis plants in the wild-type background (Fig. 2c), the acc2 knockout produced by CRISPR–Cas9 editing greatly enhanced the selection efficiency of transplastomic events (Table 1). This is largely due to suppression of undesired background growth of untransformed callus material that hinders proliferation of transplastomic cells (Supplementary Fig. 4).

Combination of the At-Δacc2 recipient line with our root-based tissue culture and selection system produced transplastomic events at high frequency and solved the infertility problem inherent in leaf-based regeneration systems. The transformation frequency determined (of approximately one transplastomic event per three shots; Table 1) is by far high enough for routine use of the technology for plastid genome engineering in Arabidopsis. Although the root tissue-based method developed here is more laborious and time consuming than the leaf-based plastid transformation protocol that is useable in the well-established tobacco system, the early attainment of homoplasmy and the non-necessity to conduct additional regeneration rounds partly offset these extra investments in time and work.

Importantly, the acc2 knockout alleles produced as recipient lines for chloroplast transformation do not cause any visible phenotype, consistent with the presence of natural null alleles in some Arabidopsis ecotypes\textsuperscript{26,27}. Nonetheless, in certain cases, it may be desirable to obtain transplastomic lines in the ACC2 wild-type background. Taking advantage of the maternal inheritance of plastids\textsuperscript{25,46}, this can be readily achieved by reintroducing the ACC2 allele into transplastomic lines through a simple cross. Similarly, the transfer of transgenic plastids to other ecotypes of Arabidopsis can easily be done by crossing.

The development of a workable method for chloroplast genome engineering closes a large gap in the spectrum of technologies...
Plants in soil were grown under standard greenhouse conditions (16h day length; temperature regime: 21 °C during the day and 18 °C at night) at an average light intensity of 300 µE m⁻² s⁻¹.

Construction of plastid transformation vectors. Plasmids pCH8, pF1151 and pF1153 (GenBank accession numbers MH590891, MH590893 and MH590894, respectively) are all based on pBluescript II SK (+). The homologous sequences for transformation of the *A. thaliana* chloroplast genome were inserted between the *KpnI* and *EcOrI* sites. The codon-optimized *aadA* sequence present in pF1151 and pF1153 was chemically synthesized (MWG-Biotech). The three constructs were created by consecutive rounds of classical cloning using type II restriction endonucleases and DNA ligase. The intergenic region between *trnM* and *trnP* was chosen as the transgene integration site (Fig. 2a), and the corresponding flanking sequences for homologous recombination were amplified from the *A. thaliana* chloroplast genome (NC_009321.1: nucleotide positions 36,604 and 36,606–37,895) by PCR. The selectable marker gene cassette (conferring spectinomycin resistance) was generated by combining the maize *cpIP* promoter and 5′ UTR TR, the *aadA* coding sequence from *E. coli* (pCH8) or the synthetic version codon optimized for *A. thaliana* chloroplasts (pF1151 and pF1153), and the *rps16* terminator from *N. tabacum* (pCH8) or the *rnsB* terminator from *E. coli* (pF1151 and pF1153; Fig. 2a). The psaA promoter and atp8 terminator from the C. reinhardtii chloroplast genome were used for expression of the yfp gene. Plastid transformation vectors pSTS5, pSTS7, pSTS10, pSTS12, pSTS13 and pCH7 are based on the same vector backbone, and their transgenes and expression elements are listed in Table 1. Vector pSTS7 harbours an *aadA* cassette developed for tobacco plastids and pSTS7 harbours a cassette designed for very high-level *aadA* expression, pSTS10 and pSTS12 are derived from pSTS5 and pSTS7, respectively, but additionally contain a gfp cassette. pSTS7 and pCH7 contain an *apha-6* cassette conferring kanamycin resistance as a selectable marker gene.

Construction of a vector for CRISPR–Cas editing of ACC2. Plasmid pHEE2E-TRI was ordered from Addgene (plasmid number 71288; http://www.addgene.org/71288/) and used for the cloning of CRISPR–Cas9 vectors. The plasmid harbours a cas9 gene driven by an egg cell-specific promoter, thus minimizing off-target effects while editing the target locus with high efficiency. To facilitate easy exchange of the gene-specific single guide RNA (sgRNA) protospacer sequences, an intermediate vector (pJF1031) was constructed in which the sequence between the U6-26 promoter and the second sgRNA scaffold was replaced by two BsaI sites. To this end, the U6-26 promoter from pHEE2E-TRI (flanked by HindIII and Ascl sites) was amplified with primers oJF212 and oJF213, and the sgRNA scaffold from the U6-26 terminator and from pHEE2E-TRI (flanked by Ascl and SpeI sites) were amplified with primers oJF214 and oJF215 (Supplementary Table 1). The PCR products were then digested with HindIII/Ascl/SpeI and inserted back into the pHEE2E-TRI backbone cut with HindIII and SpeI. The final plasmid pF1046 was created by amplifying the sgRNA scaffold, U6-26 terminator and U6-29 promoter from pHEE2E-TRI with primers oF217 and oF218 (flanked by BsaI sites and adding an sgRNA target sequence at each side). Both protospacers target the *A. thaliana* ACC2 (Atig36180) locus, the sequence of the first being 5′-CATGAGATATATTGGTCG-3′, and that of the second being 5′-GATGATTCCCGTGAAGATG-3′ (Supplementary Fig. 5). The resulting PCR product was cloned into pF1031 in a simultaneous digestion (BsaI) and ligation reaction. The final transformation vector pF1046 is identical to pHEE2E-TRI except for the two protospacer sequences. The two anti-ACC2 (Atig36180) sgRNA sequences used to construct pF1046 were selected to have no potential off-target site in the (sequence-related) upstream ACC1 gene (Atig36160).

**Agrobacterium-mediated** nucleoid transformation of *Arabidopsis* and genotyping of transgenic plants. *A. thaliana* ecotype C24 plants were transformed by floral dip transformation using *Agrobacterium tumefaciens* strain GV3101 pmp99 harbouring vector pF1046. Transgenic T1 seedlings were selected on synthetic Murashige and Skoog (MS) medium (1/2 MS + 1% sucrose) containing 25 mg/l hygromycin. For genotyping of transgenic lines, the genomic ACC2 sequence surrounding the sgRNA binding sites was amplified with primers oF324 and oF325, yielding a PCR product of 752 base pairs (bp) in *A. thaliana* C24 wild-type plants, and a 243-bp product for the deletion allele. To characterize the inversion allele (Supplementary Fig. 5b), primer oF325 was combined with primer oF219, amplifying a fragment of 195 bp.

**Biolistic transformation and regeneration of transgenic and plastidic plants.** Tissue culture media and regeneration procedures were adapted with modifications from previously published protocols for the nuclear transformation of *Arabidopsis* roots with *Agrobacterium*. All chemicals were purchased from Duchefa Biochemie. Medium AtGM was used for the generation of root material for transformation, and contains half-concentrated MS salts, 1% sucrose and 0.5 g l⁻¹ 2-(N-morpholino)ethanesulfonic acid (MES) monohydrate. The pH was adjusted to 5.7, and the medium was solidified with 0.68% Micro agar. Seeds were germinated on netting (polyester netting PES51 cut to circles of ~80 mm

Methods

**Plant material, growth conditions and genetic crosses.** *A. thaliana* ecotype C24 was used for all of the experiments. Root material for the transformation experiments was harvested from seedlings raised under aspecic conditions from surface-sterilized seeds on synthetic medium (see below) at an average light intensity of 75 µE m⁻² s⁻¹. The day length was 12 h (at 20 °C), followed by a 12 h dark period at 18 °C.

To conform the homoplasmic state of the plastidomic lines and maternal transgene inheritance, plastidic plants were selfed and reciprocally crossed with wild-type plants. Seeds were harvested and assayed by germination on spectinomycin-containing (100 mg l⁻¹) AtGM medium (see below).

![Fig 5 | Immunoblot analysis of YFP accumulation in transplastomic Arabidopsis plants.](image-url) Two independently generated transplastomic At-Δa*-JF1151 lines were analysed. The wild type (At-WT) and a transplastomic At-Δa*-CH8 line were included as negative controls. Samples of total soluble protein extracted from leaves (with the amounts given in µg) were separated by polyacrylamide gel electrophoresis and blotted. A dilution series of YFP purified from *E. coli* (with the amounts given in ng) was loaded for semiquantitative assessment of protein accumulation in transplastomic plants. Note that the YFP recombinantly expressed in bacteria migrates slightly slower than the YFP in the transplastomic samples. This is due to the presence of a purification tag (His-tag) in the YFP isolated from *E. coli* (see Methods for further details). These experiments were repeated independently twice with similar results.
AtSIM3 (in a 16 h light at 25 °C and 8 h dark at 20 °C regime). AtSIM3 consists of Gamborg B5 salts supplemented with McCown Woody plant vitamin mixture, 2.2% glucose monohydrate, 0.5 g/l MES monoxydride, 0.05 mg l−1 kinetin, 0.5 mg l−1 2,4-dichlorophenoxyacetic acid and 30 mM α-phospholipoxine. The pH was adjusted to 5.7 and the medium solidified with 0.54% Daishin agar.

For the generation of regenerated plantlets and root induction, shoots were transferred to medium ATRIM for 14d (Supplementary Fig. 7). This consists of MS salts supplemented with MS vitamins, 1% sucrose, 0.5 g/l MES monoxydride and 1 mg/l 1–3-indolebutyric acid. The pH was adjusted to 5.7 and the medium solidified with 0.65% Daishin agar.

Bioliost transformation experiments were performed with a helium-driven particle gun (PDS-1000/He; Bio-Rad) using DNA-coated gold particles of 0.6 μm diameter and 1,800 psi rupture disks. Samples were bombarded one to three times.

For the nuclear transformation experiments, vector pGreenII 0029 with a gfp transgene driven by CaMV 3SS expression elements was used15.

Isolation of nucleic acids and gel blot analyses. Total plant DNA was extracted using a cetyltrimethylammoniumbromide-based method 16. Southern blot analysis, DNA samples were digested with the restriction enzyme BglII, followed by separation of the fragments by electrophoresis in 1% agarose gel and blotting onto Hybond-XL membranes (GE Healthcare). A hybridization probe was prepared by PCR amplification with primers oSR2 and oSR1 (Supplementary Table 1) and purification by agarose gel electrophoresis using the NucleoSpin Extract II kit (Macherey-Nagel). Probes were labelled with [α-32P]dCTP by random priming (GE Healthcare), and hybridizations were performed at 65°C in Rapid-Hyb buffer (GE Healthcare) according to the manufacturer’s protocol.

Fluorescence imaging and microscopic analyses. To visualize the YFP fluorescence of transplastomic seedlings growing in Petri dishes (Fig 4a), the entire Petri dish was scanned with an Amersham Typhoon RRGB Biomolecular Imaging System (GE Healthcare) using an 884 nm laser for excitation and the Cy2 Filter (525BP20) for detection of YFP fluorescence, and 635 nm laser light for excitation and the Cy5 Filter (670BP30) for detection of chlorophyll fluorescence. The subcellular localization of YFP fluorescence was determined by confocal laser-scanning microscopy (TCS SP5; Leica) using an argon laser for excitation (at 514 nm), a 524–566 nm filter for the detection of YFP fluorescence and a 650–700 nm filter for the detection of chlorophyll fluorescence.

Protein extraction and immuno blot analysis. Total soluble protein from leaf tissue samples was extracted in 250 μl lysis buffer (50 mM HEPES/KOH pH 7.5, 10 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM DTT and 1× protease inhibitor cocktail (Roche)). Gel electrophoretic separation and immunoblot analysis were done according to standard protocols using a dilution series of recombinantly expressed YFP (purified from E. coli) for semiquantitative assessment of protein accumulation17. A commercial anti-GFP antibody (Living Colors A.v. Monoclonal Antibody [IL-8]; Takara Bio; Clontech Laboratories) was used for detection.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data supporting the findings of this study are available within the paper and its Supplementary Information files. Annotated sequences of plasmid transformation vectors pH8, pH115 and pH1153 have been deposited in GenBank (accession numbers MH590891, MH590893 and MH590894). Nature Plants thanks Francis Quétier, Spencer M. Whitney and other anonymous reviewers for their contributions to the peer review of this work.

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Author contributions

S.R., J.F. and R.B. designed the research. C.H., X.K., S.S., A.S., L.S. and J.F. performed the experiments. All authors participated in data evaluation. R.B. wrote the manuscript, with input from S.R. and J.F.

Competing interests

The authors declare no competing interests.

Additional information

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- The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement
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- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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The data supporting the findings of this study are available within the paper and its supplementary information files. Annotated sequences of plastid transformation vectors pCH8, pJF1151 and pJF1153 were deposited in GenBank (accession numbers MH590891, MH590893 and MH590894).
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | The sample sizes are given where appropriate. A sufficiently large number of samples was bombarded to guarantee isolation of at least three independent transplastomic lines from all transformation experiments with our optimized protocol. |
| Data exclusions | No data were excluded. |
| Replication | All independent plastid transformation experiments with our optimized protocol were successful. All experiments were repeated and gave similar results. The number of replicates is given in the figure legends. |
| Randomization | No randomization was done. It was not required, because the generated transplastomic lines were independently confirmed by molecular methods and inheritance assays. |
| Blinding | No blinding was done. It was not required, because the generated transplastomic lines were independently confirmed by molecular methods and inheritance assays. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study | n/a |
|-------|----------------------|-----|
| | Unique biological materials | |
| | Antibodies | |
| | Eukaryotic cell lines | |
| | Palaeontology | |
| | Animals and other organisms | |
| | Human research participants | |

Methods

| n/a | Involved in the study |
|-----|----------------------|
| | ChIP-seq |
| | Flow cytometry |
| | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

Unique materials (acc2 CRISPR/Cas lines, plastid transformation vectors) are available from the authors.

Antibodies

Antibodies used

anti-GFP antibody: Living Colors® A.v. Monoclonal Antibody (JL-8), Takara Bio, Clontech Laboratories, Mountain View, CA; catalog number 632381, clone name: JL-8, lot number: A5033481; dilution used: 1:1000

Validation

The anti-GFP antibody used is a standard commercial antibody. It was validated by the supplier (see https://www.takarabio.com/products/antibodies-and-elsa/fluorescent-protein-antibodies/green-fluorescent-protein-antibodies?catalog=632475) as follows: “The quality and performance of this lot of Living Colors A.v. Monoclonal Antibody (JL-8) was tested by Western blot analysis using lysate made from a HEK 293 cell line stably expressing AcGFP1. After cells were collected and lysed using SDS sample buffer, the lysate (10 μl; equivalent to 35,000 cells) was electrophoresed on a 12% SDSpolyacrylamide gel and transferred to a nitrocellulose membrane. The blot was probed with the Living Colors A.v. Monoclonal Antibody, JL-8 (diluted 1:1,000), followed by a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (HRP). The HRP signal was detected by chemiluminescence. A band of approximately 30 kDa corresponding to AcGFP1 was observed in the lane loaded with the AcGFP1 cell lysate. A band of this size was not detected in the lysate of untransfected HEK 293 cells.” The specificity of the antibody in our study was further confirmed by...
inclusion of negative controls (plants not harboring the yfp transgene).