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High-efficiency generation of fertile transplastomic *Arabidopsis* plants

Stephanie Ruf, **Joachim Forner**, **Claudia Hasse**, **Xenia Kroop**, **Stefanie Seeger**, **Laura Schollbach**, **Anne Schadach**, and **Ralph Bock***
Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, D-14476 Potsdam-Golm, Germany

Abstract

The development of technologies for the stable genetic transformation of plastid (chloroplast) genomes has been a boon to both basic and applied research. However, the 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 source tissue for biolistic transformation. The method produces fertile transplastomic plants at high frequency when combined with a CRISPR/Cas9-generated knock-out 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 ago1,2. Since then, the technology has proven highly valuable in both basic research and biotechnology3–6. It paved the way to the study of all steps in plastid gene expression and their regulation *in vivo*, facilitated the systematic investigation of the functions of chloroplast genes and open reading frames by reverse genetics7,8 and enabled the

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***Correspondence and requests for materials** should be addressed to Ralph Bock, Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, D-14476 Potsdam-Golm, Germany, Tel.: +49(0)331-567-8700, Fax: +49(0)331-567-8701, rbock@mpimp-golm.mpg.de.

Data availability
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).

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.

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experimental reconstruction of key processes in genome evolution9–11. In addition, the transplastomic technology opened up new applications in metabolic engineering12, molecular farming13,14 and resistance engineering15,16.

Given the transformative nature of the technology in so many research areas, enormous efforts have been made in both the academic and the commercial sectors to extend the chloroplast transformation technology to other algae and plants, most importantly model species and agriculturally important crops3,5,17. 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 species18,19, lettuce20 and poplar21. What all these plants have in common is that they display favorable properties in *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 the expansion of the species range of the transplastomic technology.

For the above reasons, plastid transformation has proven to be a serious challenge also 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 in 1998 (ref. 22), 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 efficient23, but has not solved the fertility problem24. 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 (agroinfection of roots, vacuum infiltration of flowers, 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 knock-out of a nuclear locus that determines sensitivity to the selection agent spectinomycin26,27, 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 likely will enable new synthetic biology applications in *Arabidopsis* chloroplasts28.

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

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roots29. Protocols for nuclear transformation of *Arabidopsis* root tissue had been developed30 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 *Arabidopsis thaliana* C24, a standard ecotype that is widely used, for example, in research on abiotic and biotic stresses31, and in studies on the molecular and physiological basis of heterosis32. We revived the protocols for nuclear transformation of *Arabidopsis* roots30, and modified them for biolistic transformation and spectinomycin selection of transplastomic cells (see Methods; Supplementary Figs. 1-3; Fig. 1). We used roots harvested from a lawn of young seedlings raised on synthetic medium as starting material (Supplementary Fig. 1). Alterations in the hormone composition (i.e., reduction of the concentration of 2-isopentenyladenine to 2 mg/L 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 selectable marker were conducted to optimize the parameters of the biolistic bombardment and the selection and regeneration conditions (see Methods; Supplementary Fig. 3). The optimized system produced nuclear transgenic lines at high frequency (on average 5 to 10 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 genome33,5. Antibiotic sensitivity tests revealed that *Arabidopsis* cells are much more sensitive to spectinomycin than tobacco cells and bleached out completely when exposed to concentrations as low as 5 μg/mL spectinomycin. However, consistent with previous findings34,26,27, 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 μg/mL were found to suppress 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 to be not possible (Supplementary Fig. 4).

Vector development and *Arabidopsis* chloroplast transformation

Based on previously developed vectors for plastid transformation of solanaceous species18, 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 spectinomycin33, whereas *aphA-6* confers resistance to kanamycin35. Since plastid gene expression is generally much less active in non-green tissues than in photosynthetically active leaf tissue36–38, we also constructed marker cassettes with expression signals that had been optimized for high-level expression in root plastids39. In these vectors (Table 1; Fig. 2a), the marker gene is driven by the promoter and 5’ UTR from the plastid *clpP* gene that were further enhanced by inclusion of the strong Shine-Dalgarno sequence from the *gene10* leader sequence of

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bacteriophage T7 (refs. 39–41). To further optimize marker expression, we also constructed an *aadA* expression cassette with a synthetic codon-optimized *aadA* gene. In this cassette, the *rrnB* terminator sequence (T*rrnB*) from *Escherichia coli* was used as 3' UTR (Fig. 2a). This sequence was reported to confer higher transcript stability than plastid 3' UTRs (ref. 42). As visual reporters of transgene expression, chimeric *yfp* and *gfp* cassettes were additionally incorporated into some vectors (Fig. 2a; 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 1350 biolistic bombardments (Table 1), a single transplastomic event was obtained (Fig. 2b; Supplementary Fig. 4). The line was obtained with vector pCH8 that carries the spectinomycin resistance gene *aadA* driven by the *clpP*-derived expression signals optimized for expression in non-green tissues39. This promoter-marker gene combination was, therefore, used in all subsequent transformation experiments.

The transplastomic line At-CH8-1 was characterized by Southern blot analysis (Fig. 2), which confirmed its transplastomic status and revealed homoplasmy (i.e., 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* knock-out alleles by CRISPR/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 likely prevented the successful outgrowth of transplastomic cell lines (Fig. 2b; Supplementary Fig. 4). Recent work has revealed that the sensitivity of *Arabidopsis* to inhibition of chloroplast translation is largely determined by the nuclear *ACC2* locus26,27. *ACC2* encodes an alternative acetyl-CoA carboxylase (ACCase) that, upon 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 harbor *ACC2* alleles with low activity (or null alleles) display enhanced sensitivity to spectinomycin26,27, but do not show favorable 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 plants23,24.

To improve the recovery rate of transplastomic events in our plastid transformation system, we set out to generate *acc2* null alleles in the *Arabidopsis thaliana* ecotype C24 with the help of the CRISPR/Cas9 system for genome editing43. Several putative knock-out 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 one 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

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(while the C24 wild type continued to proliferate), thus strongly suggesting that both alleles represent functional *acc2* knock-out alleles (Supplementary Fig. 5c).

To produce suitable (non-transgenic) recipient lines for plastid transformation, the *cas9* gene was segregated out and homozygous *acc2* knock-out mutants were produced for both alleles. These transgene-free lines (subsequently referred to as At-Δacc2 recipient line) were used for all subsequent plastid transformation experiments. Consistent with previous reports26,27, our *acc2* knock-out 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 ACCase in the plastid.

Greatly enhanced plastid transformation efficiency with *acc2* recipient lines

We next used root-derived microcalli from the At-Δacc2 line as 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 tissue39: pCH8 harboring 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; 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 likely representing spontaneous spectinomycin-resistant mutants44, the transplastomic status of all lines analyzed could be confirmed by PCR assays and/or DNA gel blot analyses (Fig. 2d; Table 1). When compared to transformation experiments with the wild type, growth of untransformed callus tissue was much more strongly suppressed by spectinomycin in the At-Δacc2 tissue (Fig. 2b,c; Supplementary Figs. 4 and 6), indicating that the *ACC2* gene knock-out 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 that is 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 occurring at all, this should be a rather rare event and that 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 (Fig. 2d), 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

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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 amounts of seeds (Fig. 1b; Supplementary Figs. 7 and 8). To ultimately confirm homoplasmy, 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 maternal parent. By contrast, crosses with the transplastomic plant as paternal parent (and the wild type as maternal parent) yielded a uniform population of antibiotic-sensitive seedlings, in line with the maternal inheritance of the plastid genome44–46.

The successful production of transplastomic At-Δa-JF1151 plants, that additionally carry a *yfp* cassette, enabled us to (i) also test for uniparental inheritance of the fluorescent reporter, and (ii) analyze the 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 to 2% of the total soluble protein (Fig. 5).

Discussion

Our work reported here has overcome the main obstacle that had prevented the extension of the transplastomic technology to the model plant *Arabidopsis 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 produced22,23. As noted previously24, the major “challenge is therefore to identify procedures and ecotypes that facilitate conversion of transplastomic callus of Arabidopsis into stable and heritable plant material”.

The key to our success with the establishment of a workable transplastomic protocol for *Arabidopsis* were (i) 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 (ii) the knock-out of the nuclear *ACC2* locus that had been shown previously to determine the tolerance of *Arabidopsis* cells to spectinomycin26,27, thus facilitating the selection of spectinomycin-resistant cells23. Although we were also able to obtain fertile transplastomic *Arabidopsis* plants in the wild-type background (Figure 2c), the

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acc2 knock-out produced by CRISPR/Cas 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 1 transplastomic event per 3 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 nonnecessity to conduct additional regeneration rounds partly offset these extra investments in time and work.

Importantly, the *acc2* knock-out 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* ecotypes26,27. 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 plastids44–46, this can be readily achieved by reintroducing the *ACC2* allele into transplastomic lines through a simple cross. Similarly, 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 available for the model plant *Arabidopsis*. The technology will be particularly useful to study plastid-nuclear interactions and to set up genetic screens for factors that regulate plastid gene expression at all levels (transcription, RNA processing and turnover, translation, protein stability and assembly). This has not been possible in tobacco, the species currently used for chloroplast transformation experiments, because tobacco is an allotetraploid and lacks sufficiently developed resources for nuclear genetics.

Given the growing interest in using the plastid as a chassis for synthetic biology and synthetic genomics28,47,48 the availability of a transplastomic technology will also enable synthetic biology applications in *Arabidopsis*. Moreover, the development of an efficient method for biolistic transformation of *Arabidopsis* will facilitate new transgenic applications in the nucleus, especially multigene engineering approaches such as combinatorial transformation (a large-scale co-transformation approach depending on the introduction of multiple plasmids) that cannot be done by *Agrobacterium*-mediated transformation methods49,50.

Finally, the protocols developed in the course of this work will inform approaches to develop plastid transformation technology for other species, especially the many plant species that are recalcitrant to efficient selection and regeneration of transgenic and transplastomic cells from leaf explants.

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Methods

Plant material, growth conditions and genetic crosses

Arabidopsis thaliana ecotype C24 was used for all experiments. Root material for transformation experiments was harvested from seedlings raised under aseptic 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 an 12 h dark period at 18°C.

To confirm the homoplasmic state of transplastomic lines and maternal transgene inheritance, transplastomic plants were selfed and reciprocally crossed to wild-type plants. Seeds were harvested and assayed by germination on spectinomycin-containing (100 mg/L) AtGM medium (see below).

Plants in soil were grown under standard greenhouse conditions (16 h daylength, temperature regime: 21°C during the day, 18°C at night) at an average light intensity of 300 μE m^{-2} s^{-1}.

Construction of plastid transformation vectors

Plasmids pCH8, pJF1151 and pJF1153 (GenBank accession numbers MH590891, MH590893 and MH590894) are all based on pBlueScript II SK (+). The homologous sequences for transformation of the *A. thaliana* chloroplast genome were inserted between the KpnI and EcoICRI sites. The codon-optimized *aadA* sequence present in pJF1151 and pJF1153 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 *trnfM* and *trnG* was chosen as transgene integration site18 (Fig. 2a) and the corresponding flanking sequences for homologous recombination were amplified from the *A. thaliana* chloroplast genome (NC_000932.1, nucleotide positions 35,431-36,604 and 36,606-37,895) by PCR. The selectable marker gene cassette (conferring spectinomycin resistance) was generated by combining the maize *clpP* promoter and 5' UTR (ref.39), the *aadA* coding sequence from *E. coli* (pCH8) or the synthetic version codon optimized for *A. thaliana* chloroplasts (pJF1151, pJF1153), and the *rps16* terminator from *N. tabacum*51 (pCH8) or the *rrnB* terminator from *E. coli*42 (pJF1151, pJF1153; Fig. 2a). The *psaA* promoter and *atpB* terminator from the *Chlamydomonas reinhardtii* chloroplast genome were used for expression of the *yfp* gene52. 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 pSTS5 harbors an *aadA* cassette developed for tobacco plastids33 and pSTS7 a cassette designed for very high-level *aadA* expression40. pSTS10 and pSTS12 are derived from pSTS5 and pSTS7, respectively, but additionally contain a *gfp* cassette. pSTS13 and pCH7 contain an *aphA-6* cassette53 conferring kanamycin resistance as selectable marker gene.

Construction of a vector for CRISPR/Cas editing of *ACC2*

Plasmid pHEE2E-TRI (ref. 43) was ordered from Addgene (plasmid no. 71288; https://www.addgene.org/71288/) and used for cloning of CRISPR/Cas9 vectors. The plasmid

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harbors a *cas9* gene driven by an egg cell-specific promoter, thus minimizing off-target effects while editing the target locus with high efficiency43. To facilitate easy exchange of the gene-specific 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 AscI sites) was amplified with primers oJF212 and oJF213, and the sgRNA scaffold and the U6-26 terminator from pHEE2E-TRI (flanked by AscI and SpeI sites) were amplified with primers oJF214 and oJF215 (Supplementary Table 1). The PCR products were then digested with HindIII/AscI/SpeI and inserted back into the pHEE2E-TRI backbone cut with HindIII and SpeI. The final plasmid pJF1046 was created by amplifying the sgRNA scaffold, the U6-26 terminator and the U6-29 promoter from pHEE2E-TRI with primers oJF217 and oJF218 (flanked by BsaI sites and adding an sgRNA target sequence at each side). Both protospacers target the *Arabidopsis thaliana ACC2* (At1g36180) locus, the sequence of the first one being 5'-CCATGGAGATATATTCGTG-3', and that of the second one 5'-GTAGTACCCGGTAGAAATG-3' (Supplementary Fig. 5). The resulting PCR product was cloned into pJF1031 in a simultaneous digestion (BsaI) and ligation reaction. The final transformation vector pJF1046 is identical to pHEE2E-TRI except for the two protospacer sequences. The two anti-*ACC2* (At1g36180) sgRNA sequences used to construct pJF1046 were selected to have no potential off-target site in the (sequence-related) upstream *ACC1* gene (At1g36160).

Agrobacterium-mediated nuclear transformation of *Arabidopsis* and genotyping of transgenic plants

Arabidopsis thaliana ecotype C24 plants were transformed by floral dip transformation using *Agrobacterium tumefaciens* strain GV3101 pmp90 harboring vector pJF1046. Transgenic T1 seedlings were selected on synthetic medium (1/2 MS + 1 % sucrose54) containing 25 mg/L hygromycin.

For genotyping of transgenic lines, the genomic *ACC2* sequence surrounding the sgRNA binding sites was amplified with primers oJF324 and oJF325, yielding a PCR product of 752 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 oJF325 was combined with primer oJF219 amplifying a fragment of 195 bp.

Biolistic transformation and regeneration of transgenic and transplastomic plants

Tissue culture media and regeneration procedures were adapted with modifications from previously published protocols for nuclear transformation of *Arabidopsis* roots with *Agrobacterium*30. All chemicals were purchased from Duchefa Biochemie B.V.

Medium AtGM was used for generation of root material for transformation, and contains half-concentrated MS salts54, 1 % sucrose and 0.5 g/L 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 PES1 cut to circles of ~80 mm diameter; mesh size 210 μm, A. Hartenstein GmbH, Würzburg, Germany). After harvest (Supplementary Fig. 1), the root tissue was exposed to medium AtCIM1 for

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microcallus induction in the dark (under a diurnal temperature cycle of 25°C for 16 h and 20°C for 8 h). AtCIM1 consists of Gamborg B5 salts supplemented with McCown woody plant vitamin mixture, 2.2 % glucose monohydrate, 0.5 g/L MES monohydrate, 0.05 mg/L kinetin, 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4 D), 30 nM α-phytosulfokine55 (α-PSK). The pH was adjusted to 5.7 and the medium solidified with 0.54 % Daishin agar. Selection of transgenic and transplastomic lines from bombarded tissue was conducted on medium AtSIM3 (in a 16 h light at 25 °C / 8 h dark at 20 °C regime). AtSIM3 consists of Gamborg B5 salts supplemented with McCown Woody plant vitamin mixture, 2 % sucrose, 0.5 g/L MES monohydrate, 0.15 mg/L indole-3-acetic acid (IAA), 2.0 mg/L N6-[2-isopentenyl]adenine (2-iP), and 50 mg/L kanamycin for selection of nuclear transgenic lines or 10-50 mg/L spectinomycin for selection of transplastomic lines (for details, see text and Supplementary Figs. 2-4 and 6). The pH was adjusted to 5.7 and the medium solidified with 0.54 % Daishin agar. For growth of regenerated plantlets and root induction, shoots were transferred to medium AtRIM1 for 14 days (Supplementary Fig. 7). It consists of MS salts supplemented with MS vitamins, 1 % sucrose, 0.5 g/L MES monohydrate, 1 mg/L 3-indolebutyric acid (IBA). The pH was adjusted to 5.7 and the medium solidified with 0.65 % Daishin agar. Plantlets were incubated in a 12 h light/12 h dark regime at 20°C/18°C. For seed production, plants were transferred to medium AtPM1 and grown to maturity (Supplementary Fig. 8). AtPM1 consists of MS salts supplemented with MS vitamins, 1 % sucrose and 0.5 g/L MES monohydrate. The pH was adjusted to 5.7 and the medium solidified with 0.65 % Daishin agar.

Biolistic transformation experiments were performed with a helium-driven particle gun (PDS-1000He; Bio-Rad) using DNA-coated gold particles of 0.6 μm diameter and 1800 psi rupture disks. Samples were bombarded one to three times. For nuclear transformation experiments, vector pGreenII0029 with a *gfp* transgene driven by CaMV 35S expression elements was used56.

Isolation of nucleic acids and gel blot analyses

Total plant DNA was extracted using a cetyltrimethylammoniumbromide (CTAB)-based method57. For Southern blot analyses, DNA samples were digested with the restriction enzyme BglII, followed by separation of the fragments by electrophoresis in 1% agarose gels 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[^{32}P]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 YFP fluorescence of transplastomic seedlings growing in Petri dishes (Fig. 4a), the entire Petri dish was scanned with an Amersham Typhoon RGB Biomolecular Imager (GE Healthcare) using 488 nm laser light 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

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fluorescence was determined by confocal laser-scanning microscopy (TCS SP5; Leica, Wetzlar, Germany) using an argon laser for excitation (at 514 nm), a 524-566 nm filter for detection of YFP fluorescence and a 650-700 nm filter for detection of chlorophyll fluorescence.

Protein extraction and immunoblot analysis

Total soluble protein from leaf tissue samples was extracted in 250 µL lysis buffer [50 mm HEPES/KOH pH 7.5, 10 mm KAc, 5 mm MgAc, 1 mm EDTA, 1 mm DTT, 1× protease inhibitor cocktail cOmplete (Roche)]. Gel electrophoretic separation and immunoblot analysis were done according to standard protocols using a dilution series of recombinantly expressed YFP (purified from *Escherichia coli*) for semiquantitative assessment of protein accumulation52. A commercial anti-GFP antibody (Living Colors® A.v. Monoclonal Antibody JL-8, TaKaRa Bio, Clontech Laboratories) was used for detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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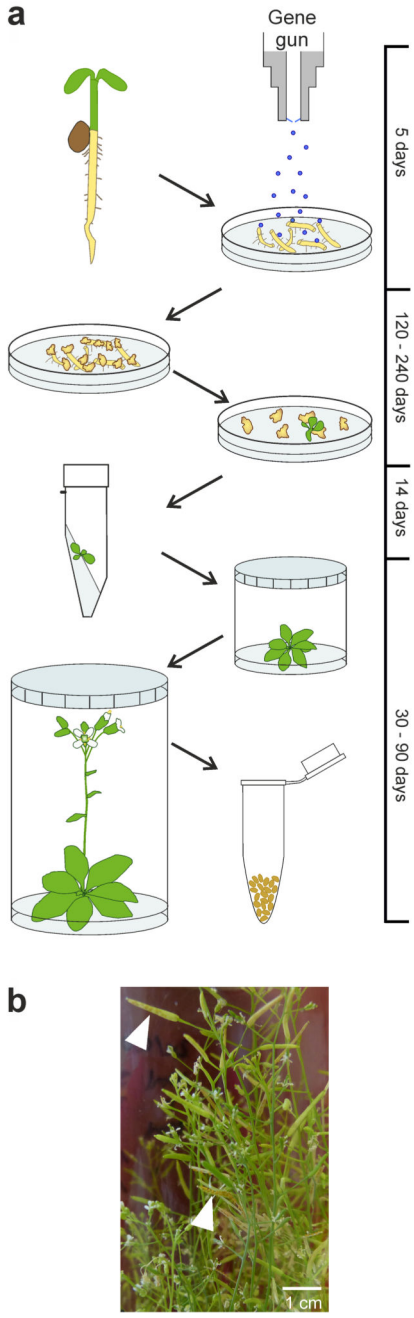

Fig. 1.

Biolistic nuclear and plastid transformation of *Arabidopsis thaliana*. (**a**) Workflow of transformation experiments. Microcalli are induced from root tissue (6 days after germination) harvested from seedlings raised on synthetic medium. Biolistic bombardment is conducted after 5 days of incubation on callus-inducing medium and followed by selection for kanamycin resistance (nuclear transformation) or spectinomycin resistance (chloroplast transformation). Resistant shoots are rooted on agar slants and plants are grown to maturity in sterile containers. A timeline indicating the approximate duration of the

individual steps in the protocol is given at the right. See text and Supplementary Figs. 1-4 and 6-8 for details. (**b**) Fertility of regenerated *Arabidopsis* plants. The plants are fertile and produce large amounts of seeds. Two ripe siliques in which the seeds can be seen are indicated by white arrowheads. These experiments were repeated independently for 22 transplastomic lines with similar results.

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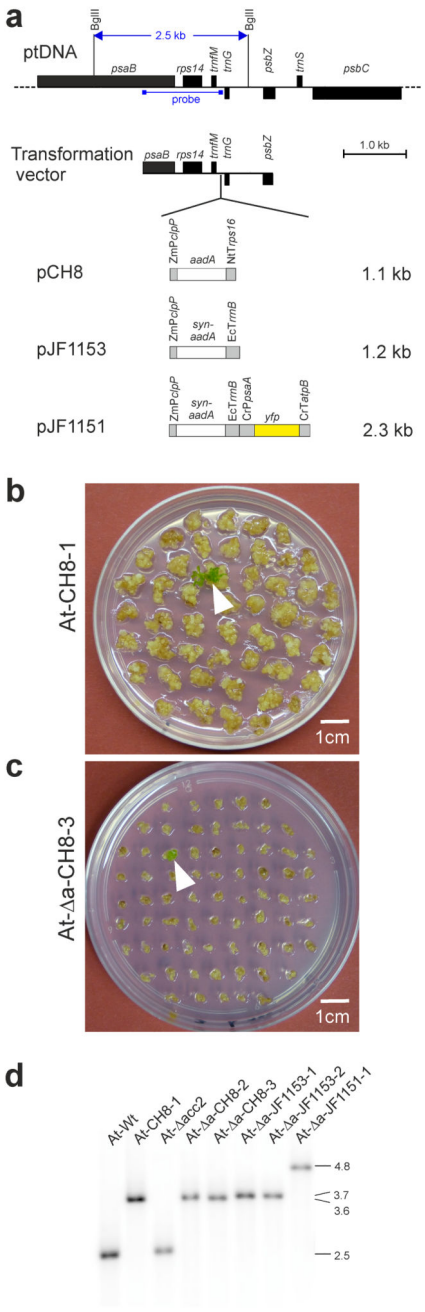

Fig. 2.

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, genes below the line are transcribed from the opposite strand of the ptDNA. The transgenes are inserted into the intergenic spacer between the *trnfM* and *trnG* genes within a cloned ptDNA fragment18 (Transformation vector). The location of two BglII restriction sites that were used for Southern blot analysis and the binding site of the hybridization probe

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is also indicated. The sizes of the transgene cassettes in the three vectors are given in kb. ZmP*clpP*: promoter of the plastid *clpP* gene from *Zea mays* (with the *clpP* 5' UTR and the *G10*-derived Shine-Dalgarno sequence form phage T7; ref. 39); *aadA*: *aadA* gene from *E. coli*; *syn-aadA*: synthetic codon-optimized *aadA* gene; NtT*rps16*: 3' UTR from the tobacco plastid *rps16* gene; EcT*rrnB*: rRNA operon terminator from *E. coli*; CrP*psaA*: promoter of the plastid *psaA* gene from *Chlamydomonas reinhardtii*; CrT*atpB*: 3' UTR of the plastid *atpB* gene from *C. reinhardtii*. (**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 (cf. Table 1), and resulted in similar background growth of the bombarded calli. (**c**) Selection of a transplastomic line (white arrowhead) after bombardment of *acc2* knock-out tissue (At-Δacc2) with vector pCH8. Note the much more efficient suppression of background callus growth from the *acc2* knock-out 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 (cf. panel a). The sizes of hybridizing fragments are indicated in kb at the right. These experiments were repeated independently three times with similar results.

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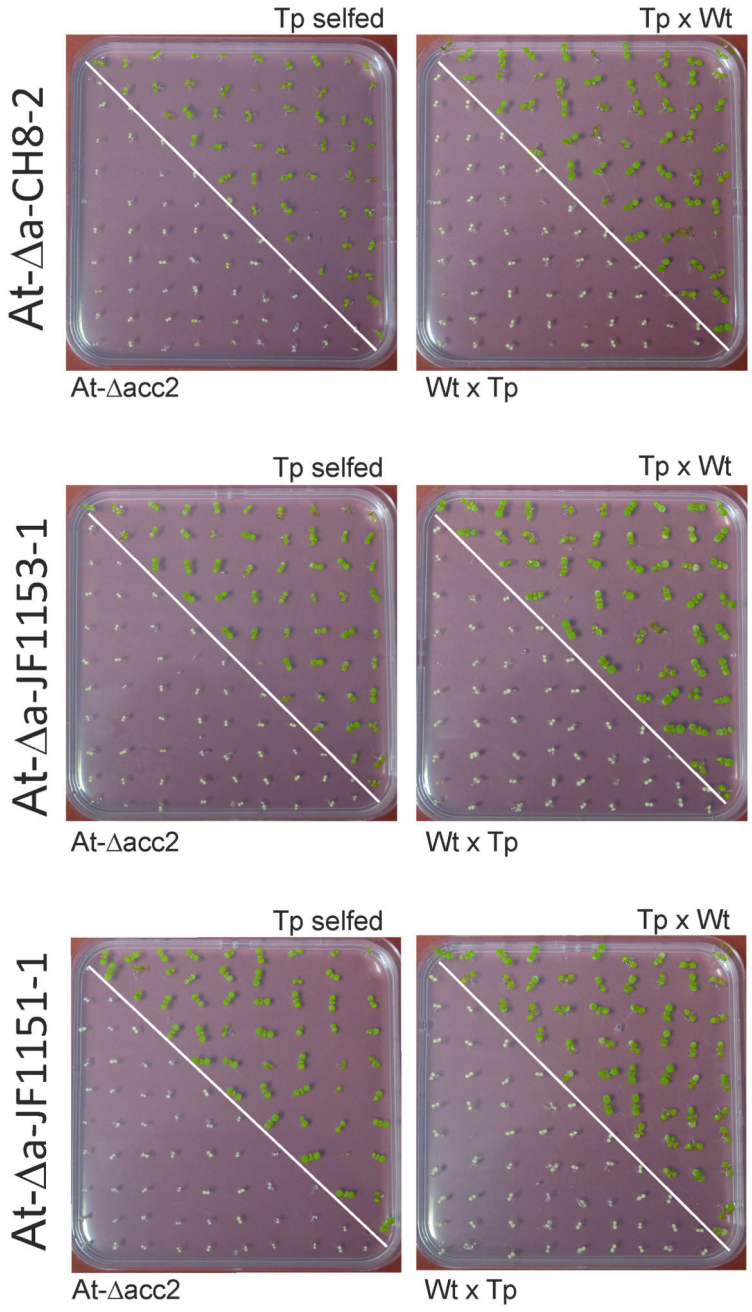

Fig. 3.

Homoplasmy of transplastomic lines obtained with vectors pCH8, pJF1153 and pJF1151, and demonstration of maternal transgene inheritance. The left plates show the comparison between the progenies of the selfed transplastomic line (Tp) and the selfed At-Δacc2 recipient line used for transformation. The right plates show the progenies of reciprocal crosses between the wild type (Wt) and the transplastomic line. Seeds were germinated in the presence of 100 mg/L spectinomycin. These assays were repeated independently two times with similar results.

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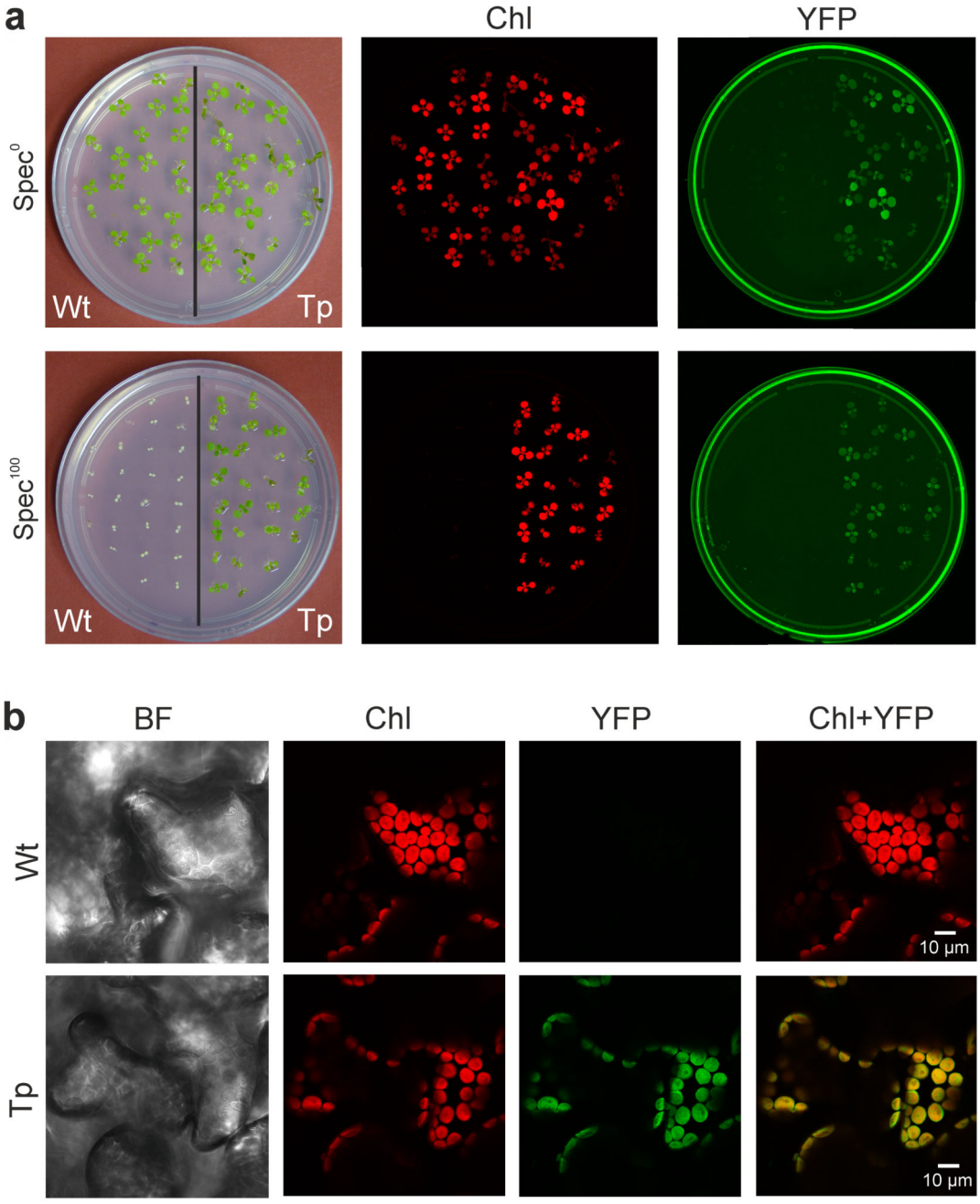

Fig. 4.

Expression of the YFP reporter in transplastomic At-Δa-JF1151 plants. (**a**) Chlorophyll and YFP fluorescence of wild-type (Wt) and transplastomic (Tp) seedlings grown in the absence ($Spec^0$) or presence ($Spec^{100}$) of spectinomycin. The plates were scanned with a Typhoon imager that produces a red image of the chlorophyll fluorescence and a green image for the (yellow) YFP fluorescence. The images were taken 20 days after sowing. (**b**) Confirmation of chloroplast YFP expression in leaf mesophyll cells by confocal laser-scanning microscopy. BF: bright-field image; Chl: chlorophyll fluorescence; YFP: YFP fluorescence

(colored in green, to match the color of the Typhoon image); Chl+YFP: overlay of the chlorophyll and YFP fluorescences. These experiments were repeated independently three times with similar results.

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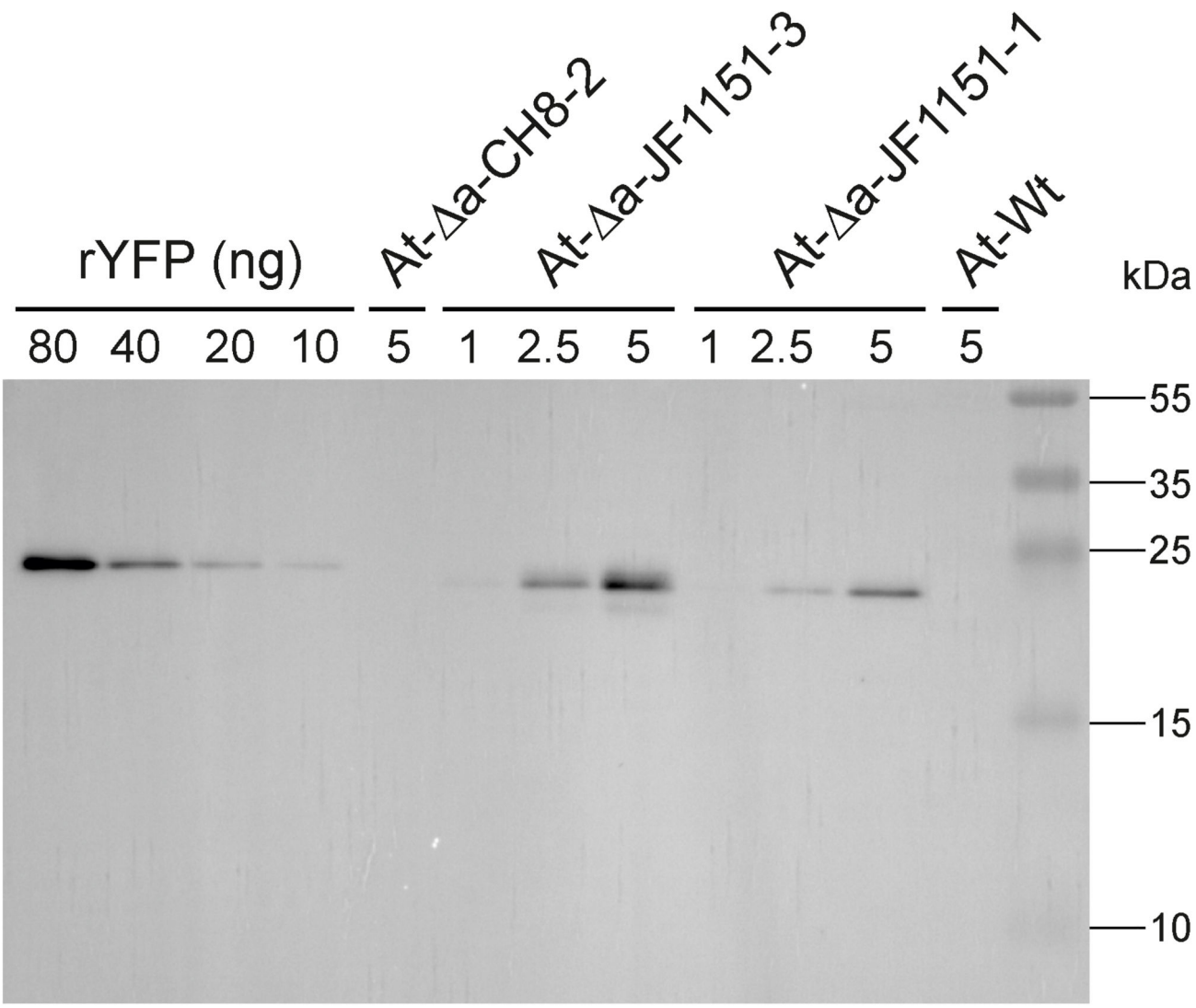

Fig. 5.

Immunoblot analysis of YFP accumulation in transplastomic *Arabidopsis* plants. Two independently generated transplastomic At-Δa-JF1151 lines were analyzed. 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 *Escherichia 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 two times with similar results.

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Table 1

Summary of transformation experiments conducted in *Arabidopsis thaliana*. Nt: *Nicotiana tabacum*; P: promoter; T: 3' UTR; *G10*: *gene10* leader from bacteriophage T7; Cr: *Chlamydomonas reinhardtii*; Zm: *Zea mays*; Ec: *Escherichia coli*.

Vector	Transgene cassettes	Selection	Recipient	Bombarded plates	Resistant lines	Resistant lines analyzed	Confirmed transplastomic lines	Spontaneous mutants	Lines grown to maturity	Lines with seeds obtained
pSTS5	NtP*rrn*.*rbcL*::*aadA*::NtT*psbA*	spec	WT	60	0	0	0	0		
pSTS7	NtP*rrn*.*G10*::*aadA*::NtT*psbA*	spec	WT	155	0	0	0	0		
pSTS10	NtP*rrn*.*rbcL*::*aadA*::NtT*psbA* Cr*PrrnG10*:*gfp*::*CrTatpA*	spec	WT	92	0	0	0	0		
pSTS12	NtP*rrn*.*G10*::*aadA*::NtT*psbA* Cr*PrrnG10*:*gfp*::*CrTatpA*	spec	WT	90	1	1	0	1		
pSTS13	NtP*rrn*.*G10*::*aphA-6*::NtT*psbA* Cr*PrrnG10*:*gfp*::*CrTatpA*	kan	WT	192	0	0	0	0		
pCH7	ZmP*clpP*.*G10*::*aphA-6*::NtT*rps16*	kan	WT	259	0	0	0	0		
pCH8	ZmP*clpP*.*G10*::*aadA*::NtT*rps16*	spec	WT	507	1	1	1	0	1	1
pCH8	ZmP*clpP*.*G10*::*aadA*::NtT*rps16*	spec	Δacc2	98	43	30	29	1	8	6
pJF1153	ZmP*clpP*.*G10*::syn*aadA*::EcT*rrnB*	spec	Δacc2	141	43	34	30	4	14	12
pJF1151	ZmP*clpP*.*G10*::syn*aadA*::EcT*rrnB* CrP*psbA*::*yfp*::CrT*atpB*	spec	Δacc2	18	6	5	5	0	3	3