



(51) International Patent Classification:

C12N 5/10 (2006.01) C12N 5/82 (2006.01)
C12N 15/09 (2006.01) C12N 9/22 (2006.01)
C12N 15/63 (2006.01) C12N 15/29 (2006.01)
C12N 15/65 (2006.01)

(21) International Application Number:

PCT/US2018/013034

(22) International Filing Date:

09 January 2018 (09.01.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/444,307 09 January 2017 (09.01.2017) US

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,
KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

(54) Title: COMPOSITIONS AND METHODS FOR IMPROVING PLASTID TRANSFORMATION EFFICIENCY IN HIGHER PLANTS

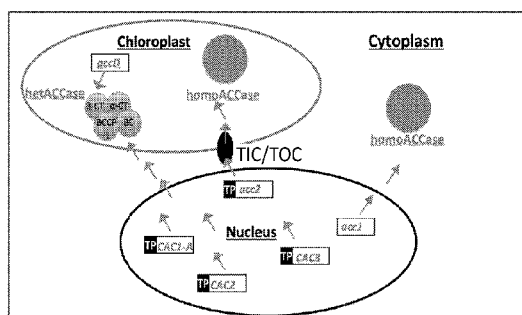


Fig. 1A

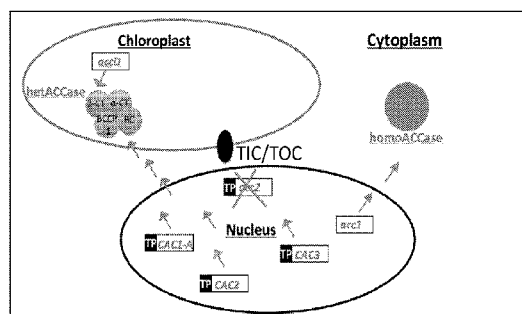


Fig. 1B

(57) Abstract: Compositions and methods for improving plastid transformation in difficult to transform plants are disclosed.

Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

Compositions and Methods for Improving Plastid Transformation Efficiency in Higher Plants

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This application claims priority under 35 U.S.C. §119(e) to U.S. Provisional Patent Application No. 62/444,307, filed on January 9, 2017. The foregoing application is incorporated by reference herein as though set forth in full.

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Field of the Invention

The present invention relates the fields of plant biology and plastid transformation. More specifically, the invention pertains to molecular strategies for improving plastid transformation efficiency in recalcitrant plant species.

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Background of the Invention

Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated by reference herein as though set forth in full.

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Plastids are semi-autonomous plant organelles with thousands of copies of the ~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 ~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. Transformation of the plastid genome in flowering plants was first accomplished in tobacco (*Nicotiana tabacum*), the current model species of plastid engineering (Svab et al., 1990; Svab and Maliga, 1993).

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Plastid transformation is routine only in tobacco, 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 ~200 events in tobacco using the technology available in 1988 (Svab and Maliga, 1993). Until now the reasons for the low efficiency in *Arabidopsis* were not understood.

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Summary of the Invention

In accordance with the present invention, a method for increasing sensitivity to spectinomycin in plastids of higher plants for increasing plastid transformation efficiency is provided. An exemplary method comprises providing a plant having a nonfunctional ACC2
10 nuclear gene, introducing one or more plastid transformation vectors into plastids in cells from said plant, said one or more vectors comprising an aadA spectinomycin resistance marker sequence and a nucleic acid sequence encoding a protein of interest. The plant cells are then contacted with spectinomycin and spectinomycin resistant plant cells which accumulate the protein of interest in said plastids selected. The method also includes culturing said plant cells
15 under conditions suitable to regenerate a transplastomic plant therefrom. In preferred embodiments, the plant is selected from the group consisting of *Arabidopsis* ssp., *Brassica* ssp., *Camelina* ssp., and *Crambe* spp. In a further aspect, the method entails excising the resistance marker from said plant. This can be achieved using the protocols provided in US Patents 8,841,511; 7,667,093 and 7,217,860.

20 Plants to be transformed can be naturally occurring ACC2 mutants which are defective in acc2 activity. Alternatively, desirable plant species can be identified and the ACC2 gene is inactivated in said plant using the CRISPR/Cas system and the appropriate guide strands.

In another embodiment, a method for seed-specific plastid expression is provided. An exemplary method comprises introducing a nuclear expression vector encoding a modified
25 PPR10 binding protein driven by a seed-specific promoter and a plastid expression vector encoding a gene of interest linked to an upstream PPR10 binding site, wherein nuclear-expressed PPR10 is imported into plastids and binds said PPR10 binding site to drive expression of the gene of interest in seed plastids. In certain embodiments, the vector comprises a seed specific promoter selected from a napin or a phaseolin gene promoter. In other embodiments, the
30 modified PPR10 binding protein is PPR10^{GG} and encoded by SEQ ID NO: 265. The PPR10 binding site may also be encoded by SEQ ID NO: 261. The vector may also comprise the

aadA spectinomycin resistance gene. Additionally, in another aspect, the plastid expressed gene of interest is linked to an upstream sequence encoding a maize *atpH* gene and/or tRNA sequence in said plastid vector.

In another aspect of the invention, a method for increasing sensitivity to plastid translation inhibitors in plastids of higher plants for increasing plastid transformation efficiency is provided. An exemplary method comprises providing a plant comprising a nonfunctional *ACC2* nuclear gene, introducing one or more plastid transformation vectors into the plastids in cells from said plant, said one or more vectors comprising a nucleic acid sequence conferring resistance to said plastid translation inhibitor, and a nucleic acid sequence encoding a protein of interest. The method further entails contacting said cells with said inhibitor and selecting plant cells which are resistant to said inhibitor and accumulate said protein of interest in said plastids; and culturing said plant cells under conditions suitable to regenerate a transplastomic plant therefrom. In certain embodiments, the plastid translation inhibitor is selected from kanamycin, chloramphenicol, tobramycin and gentamycin.

Brief Description of the Drawings

Figures 1A – 1B. Defective *ACC2* Gene Makes Chloroplasts More Sensitive to Spectinomycin. (Fig. 1A) In most accessions the 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 in figure), *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; homACCcase) enzyme localizes in the cytoplasm and the ACC2 (At1g36180; homACCcase) 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 supplies the cells with lipids so that cellular viability is not affected. (Fig. 1B) In *ACC2* mutants, the absence of the homomeric ACCase makes the plants dependent on plastid translation to produce the heteromeric ACCase enzyme for fatty acid biosynthesis.

Figure 2. 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 positions of the PrnL *atpB* promoter and the 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. The 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 analyses, respectively.

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

Figure 4. 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 and 568 nm, and detection was at 500 to 530 and 650 to 700 nm, respectively. Note the 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-0 #6 plants. Yellow color in the merged images indicates the colocalization of GFP and chlorophyll in plastids. 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. Bars = 10 μ m

Figures 5A – 5B. Molecular Characterization of the Sav-0 Transplastomic Clones. (Fig. 5A) DNA gel blot using the *rrn16* probe (Fig. 2) 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. (Fig. 5B) The *aadA* and *gfp* probes recognize the same 2 kb dicistronic mRNA.

Figures 6A – 6C. Alignment of homomeric ACCases in the Brassicaceae family. (Fig. 6A)

- 5 Alignment of 200 the N-terminal amino acids of *Arabidopsis thaliana* *ACC1* (At1g36160) and *ACC2* (At1g36180) genes. (Fig. 6B) 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*: CARUB_v10011872mg; *Brassica oleracea* *ACC1*: LOC106311006; *Brassica napus* *ACC1-1*: LOC106413885; *Brassica napus* *ACC1-2*: LOC106418889; *Brassica rapa* *ACC1*: LOC103833578. (Fig. 6C) 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_v10008063mg; *Brassica oleracea* *ACC2*: LOC106301042; *Brassica napus* *ACC2-1*: Y10302; *Brassica napus* *ACC2-2*: X77576; *Brassica rapa* *ACC2*: LOC103871500.
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Figure 7. Design of sgRNAs for simultaneous mutagenesis of both *B. napus* *ACC2* gene copies. Aligned are the first exons encoding the N-terminal plastid targeting regions. The GG of NGG of the PAM sequence is encircled; the 20 nucleotide forward guide sequence (5'-3') is marked with a horizontal line. The first nucleotide of the guide sequence should be changed to a G or an A, dependent on the use of U6 or U3 promoter, respectively (Belhaj et al., 2013). 9 of the 15 potential gRNA sequences are suitable for targeting both *ACC2* copies (2-8 and 14,15). The reverse guide sequences are included in Table 3.

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- 25 **Figure 8.** Mutations generated by CRISPR/Cas9 mutagenesis in the *Arabidopsis* Wassiliowskija (Ws) and RLD ecotypes. *Top*- Columbia reference sequence and the parental Ws/RLD sequences. Note mutations that alter the reading frame yielding non-functional protein, such as a one nucleotide insertion in Ws-6-2 and RLD-6-2 lines. *Bottom*- oligonucleotide sequence used for construction of gRNA.

Figure 9. Ws T3 seed germinated on 100 mg/L spectinomycin medium testing for hypersensitive response. After 2 weeks, the wild-type Ws seedlings bleach but develop primary leaves, in contrast to Ws-2-22 homozygous *ACC2* knock-out seedlings which germinate, but do not develop shoot meristem outgrowths on spectinomycin.

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Figure 10. Schematic design of a *Brassica napus* plastid transformation vector is shown. The plastid targeting sequence comprises the *rrn16* targeting region (nucleotides 135473-137978 in GenBank accession KP161617). The vector carries a target site flanked selectable *aadA* marker gene. The recombinase target sites are marked with triangles. The marker gene and gene of interest have different promoters (P1, P2) and terminators (T1, T2) to avoid deletions by recombination via duplicates sequences.

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Figure 11. A schematic diagram depicting system for seed-specific expression of plastid genes from *acc2* defective plants.

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Figures 12A – 12B. Transgenes for seed-specific expression in *Brassica* spp. (Fig. 12A) Plastid transgenes. P1 and T1 are the expression signals of the *aadA* marker gene. Preferred sequences are listed in text. P1 is the tobacco plastid *Prrn* sequence. The half circle is the maize sequence containing BS^{ZmGG} sequence. *gfp* encodes green fluorescence protein. T1 is the *rbcL* gene terminator. Cloverleaf symbolizes tRNA gene. (Fig. 12B) The map of *Agrobacterium* binary vector pCAMBIA2300 with the PnpaA:Zm-PPR10GG:Tocs and selectable kanamycin resistance gene. P1 and T1 are Pnos/Tnos, the expression signals of kanamycin resistance (*neo*) gene. P2 is the PnpaA napin promoter; PPR10^{GG} sequence is the mutant maize PPR10 protein coding sequence; T2 is Tocs octopine synthase transcription terminator. LB and RB are the T-DNA left and right border sequences.

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Detailed Description of the Invention

Spectinomycin, a preferred agent used for selecting for 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

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encoding aminoglycoside-3''-adenylyltransferase, the modified antibiotic 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 in tissue culture by the ability to green and regenerate shoots. In contrast, *Arabidopsis* bleaches but continues to proliferate on a spectinomycin medium in the absence of chloroplast ribosomes (Zubko and Day, 1998). Two major studies by Parker et al. (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 albino plants.

However, in certain accessions, spectinomycin blocked plant development: the seeds germinated, but did not develop beyond the cotyledonary stage. Genetic analysis revealed that spectinomycin sensitivity in these 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 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 and no heteromeric ACCase is made, the homomeric enzyme enables a limited amount of fatty acid biosynthesis and development of albino plants. 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 the inefficiency of plastid transformation observed in our early efforts with *Arabidopsis* was due to the lack of the sensitivity to spectinomycin, and that transformation of mutants defective in *ACC2* function should increase efficient recovery of transplastomic clones. We report here that the efficiency of plastid transformation in the *acc2* background in *Arabidopsis* is comparable to that of tobacco, confirming our hypothesis.

Antibiotics kanamycin, chloramphenicol, tobramycin and gentamycin are similar to spectinomycin in that they also act through inhibition of plastid translation. Kanamycin resistance is conferred by the *neo* (*nptII*) gene, encoding neomycin phosphotransferase or the *aphA-6* gene encoding an aminoglycoside phosphotransferase. Chloramphenicol resistance is conferred by the *cat* gene encoding chloramphenicol acetyltransferase. Tobramycin/gentamycin resistance is conferred by the bifunctional *aac(6')-Ie/aph(2'')-Ia* gene, abbreviated as *aac6-aph2* gene, encoding the bifunctional aminoglycoside phosphotransferase(6')-Ie/APH(2'')-Ia enzyme.

Thus, improved recovery of transplastomic events is expected in the *acc2* defective background using these inhibitors of organellar translation as selective markers.

In view of this finding, we have expanded our efforts to create additional strains of *acc2* defective plants in the Brassicaceae family. Herein below protocols and expression vectors are provided for both nuclear and plastid transformation in such plants, which include, without limitation, *A. lyrata*, *C. sativa*, *C. ruella*, *B. oleracea*, *B. napus*, *B. rapa*. The inventor also provides suitable guide strands for introducing mutations in ACCases via a CRISPR/CAS.

The definitions below are provided to facilitate an understanding of the invention.

Heteroplastomic refers to the presence of a mixed population of different plastid genomes within a single plastid or in a population of plastids contained in plant cells or tissues.

Homoplastomic refers to a pure population of plastid genomes, either within a plastid or within a population contained in plant cells and tissues. Homoplastomic plastids, cells or tissues are genetically stable because they contain only one type of plastid genome. Hence, they remain homoplastomic even after the selection pressure has been removed, and selfed progeny are also homoplastomic. For purposes of the present invention, heteroplastomic populations of genomes that are functionally homoplastomic (i.e., contain only minor populations of wild-type DNA or transformed genomes with sequence variations) may be referred to herein as "functionally homoplastomic" or "substantially homoplastomic." These types of cells or tissues can be readily purified to a homoplastomic state by continued selection.

Plastome refers to the genome of a plastid.

Transplastome refers to a transformed plastid genome.

Transformation of plastids refers to the stable integration of transforming DNA into the plastid genome that is transmitted to the seed progeny of plants containing the transformed plastids. Transient expression of heterologous DNA into the plastid or nuclear compartments can also be employed.

Selectable marker gene refers to a gene that upon expression confers a phenotype by which successfully transformed plastids or cells or tissues carrying the transformed plastid can be identified.

Transforming DNA refers to homologous DNA, or heterologous DNA flanked by

homologous DNA, which when introduced into plastids becomes part of the plastid genome by homologous recombination.

"Operably linked" refers to two different regions or two separate genes spliced together in a construct such that both regions will function to promote gene expression and/or protein translation.

"Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule of its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated. For example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism.

When applied to RNA, the term "isolated nucleic acid" refers primarily to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from other nucleic acids with which it would be associated in its natural state (i.e., in cells or tissues). An isolated nucleic acid (either DNA or RNA) may further represent a molecule produced directly by biological or synthetic means and separated from other components present during its production.

The term "functional" as used herein implies that the nucleic or amino acid sequence is functional for the recited assay or purpose.

Mao et al. provide detailed guidance for use of the CRISPR/Cas system in higher plants in *Molecular Plant*, 6: 2008–2011 (2013). The article entitled "Application of the CRISPR–Cas System for Efficient Genome Engineering in Plants" and its supplemental material is incorporated herein by reference as though set forth in full.

The terms "transform", "transfect", "transduce", shall refer to any method or means by which a nucleic acid is introduced into a cell or host organism and may be used interchangeably to convey the same meaning. Such methods include, but are not limited to, transfection,

electroporation, microinjection, PEG-fusion, biolistic bombardment and the like.

"Floral dip transformation" refers to *Agrobacterium* mediated DNA transfer, in which the flower is brought in contact with the *Agrobacterium* solution. Floral dip transformation has been described in *Arabidopsis* (Clough and Bent, 1998) and *Brassica* spp. (Verma et al., 2008; Tan et al., 2011).

"T-DNA" refers to the transferred-region of the Ti (tumor-inducing) plasmid of *Agrobacterium tumefaciens*. Ti plasmids are natural gene transfer systems for the introduction of heterologous nucleic acids into the nucleus of higher plants. Binary *Agrobacterium* vectors such as pBIN20 and pPZP222 (GenBank Accession Number U10463.1) are known in the art.

A "plastid transit peptide" is a sequence which, when linked to the N-terminus of a protein, directs transport of the protein from the cytoplasm to the plastid.

A "clone" or "clonal cell population" is a population of cells derived from a single cell or common ancestor by mitosis.

A "cell line" is a clone of a primary cell or cell population that is capable of stable growth in vitro for many generations.

A "defective" and "nonfunctional" gene, such as *acc2*, refers to a gene which does not encode a functional protein. For example, a one nucleotide insertion or deletion may alter the reading frame to create an in-frame stop codon.

METHODS FOR CREATING TRANSPLASTOMIC PLANTS USING THE COMPOSITIONS OF THE INVENTION

Virtually all dicots have *accD*, an heteromeric ACCase subunit gene encoded in their plastid genome, but also have homomeric, plastid targeted nuclear *ACC2* gene copies, which is the likely cause for the difficulty of extending the plastid transformation technology to all crops. Deletion of the nuclear *ACC2* genes will enable plastid transformation in these dicot species and genetic lines.

The recognition that the plastid targeted ACCase in *Arabidopsis* is an impediment to plastid transformation provides a rational template to implement plastid transformation in recalcitrant crops. The *accD* gene is present on the plastid genome of most crops. The *Arabidopsis thaliana* *ACC2* enzyme has an N-terminal extension relative to *ACC1* that serves as an N-terminal plastid targeting sequence (Babijchuk et al., 2011). The *ACC1* and *ACC2* genes

are present in all 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 relative to ACC1. A targeted mutation in the N-terminal extension should selectively inactivate the ACC2 variant, expected to create a spectinomycin sensitive mutant similar to the Col-0 *acc2-1* mutant derivative (Parker et al., 2014). 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, but 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), or the transformation efficiency was low (Schneider et al., 2015). Plastid transformation in *Lesquerella fendleri*, another oilseed crop in the Brassicaceae, was feasible but inefficient (Skarjinskaia et al., 2003). Mutagenesis of *ACC2* in the latter cases should significantly boost plastid transformation efficiency. Accordingly, a CRISPR/Cas approach for knocking out the ACC gene is provided in Example II.

Alternatively, desirable plant species could be screened for mutations in nuclear ACC genes and those strains harboring such mutations utilized in the plastid transformation methods disclosed herein. Such strains should inherently be more sensitive to spectinomycin.

The materials and methods set forth below were utilized in the performance of Example I.

Tissue Culture Media

The tissue culture media were adopted from Sikdar et al. (1998), originally described by Márton and Browse (1991). The culture media are based on Murashige and Skoog (MS) salts (Murashige and Skoog, 1962). ARM consists of MS salts, 3% (w/v) Suc, 0.8% (w/v) agar (A7921; Sigma), 200 mg of myoinositol, 0.1 mg of biotin (1 mL of 0.1 mg mL⁻¹ stock), and 1 mL of vitamin solution (10 mg of vitamin B1, 1 mg of vitamin B6, 1 mg of nicotinic acid, and 1 mg of Gly per mL) per liter, pH 5.8. ARM5 medium consists of ARM supplemented with 5% (w/v) Suc. ARMI medium consists of ARM containing 3 mg of IAA, 0.6 mg of benzyladenine, 0.15 mg of 2,4-D, and 0.3 mg of isopentenyladenine per liter. ARMIr medium consists of ARM supplemented with 0.2 mg/L naphthaleneacetic acid and 0.4 mg of 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 containing 2,4-D (0.5 mg/L), kinetin (0.05 mg/L), and spectinomycin (100 mg/L; 3 d) followed by incubation on an ARM containing IAA (0.15 mg/L), phenyladenine (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% (w/v) Suc and 0.8% (w/v) agar (A7921; Sigma), pH 5.8.

Plant Materials and Growth Conditions

The *Arabidopsis* (*Arabidopsis thaliana*) Sav-0 (CS28725) and Col-0 homozygous acc2-1 knockout line (SALK_148966C) seeds were obtained from the *Arabidopsis* Biological Resource Center. The Col-0 seeds were obtained from Juan Dong (Rutgers University). The RLD and Ler seeds were purchased from Lehle Seeds.

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

Plastid Transformation Vector

The plastid transformation vector pATV1 targets insertion in the inverted repeat region of the plastid genome upstream of the *trnV* gene (Fig. 2). The targeting region is a 4.5 kb *NruI/XbaI* fragment derived from the *Arabidopsis thaliana* ptDNA (GenBank Accession No. NC_000932). The fragment was cloned in the *KpnI-SacI* site of a pBSKS+ BlueScript vector, ligating the vector *KpnI* site to the plastid *NruI* site and vector *SacI* site to the plastid *XbaI* site. The vector

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). The operon is expressed from the *PrrnLatpB* promoter, obtained by fusing the plastid rRNA operon promoter (*Prrn*) with the *atpB* plastid gene leader (*LatpB*), originally described in the pHK30 plasmid (Kuroda and Maliga, 2001). The dicistronic *aadA-gfp* marker gene was excised as an *EcoRI-HindIII* fragment and cloned in the *HincII* site of the targeting region. In the dicistronic construct, 14 N-terminal amino acids of the ATP synthase beta subunit are translationally fused with the AAD N-terminus, as in plasmid pHK30 (Kuroda and Maliga, 2001). The intergenic region encodes the *cry9Aa2* gene leader (Chakrabarti et al., 2006), followed by the *gfp* coding region and the 3'-UTR of the plastid *psbA* gene (*TpsbA*) for the stabilization of the mRNA. The DNA sequence of the *EcoRI-HindIII* fragment encoding the *aadA-gfp* dicistronic operon in plasmid pMRR13 is shown below.

gagctcGCTCCCCCGCCGTCGTTCAATGAGAATGGATAAGAGGCTCGTGGGATTGACGT
 15 GAGGGGGCAGGGATGGCTATATTTCTGGGAGAATTAACCGATCGACGTGCaAGCGG
 ACATTTATTTTaAATTCGATAATTTTGTCAAAAACATTTTCGACATATTTATTTATTTA
 TTATTATGAGAATCAATCCTACTACTTCTGGTTCTGGGGTTTCCACGgctactagcGAAGC
 GGTGATCGCCGAAGTATCGACTCAACTATCAGAGGTAGTTGGCGTCATCGAGCGCC
 ATCTCGAACCGACGTTGCTGGCCGTACATTTGTACGGCTCCGCAGTGGATGGCGGCC
 20 TGAAGCCACACAGTGATATTGATTTGCTGGTTACGGTGACCGTAAGGCTTGATGAAA
 CAACGCGGCGAGCTTTGATCAACGACCTTTTGGAACTTCGGCTTCCCCTGGAGAGA
 GCGAGATTCTCCGCGCTGTAGAAGTCACCATTGTTGTGCACGACGACATCATTCCGT
 GCGGTTATCCAGCTAAGCGCGAACTGCAATTTGGAGAATGGCAGCGCAATGACATT
 CTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCTTGCTGACA
 25 AAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACCTTTTGA
 TCCGGTTCCTGAACAGGATCTATTTGAGGCGCTAAATGAAACCTTAACGCTATGGAA
 CTCGCCGCCGACTGGGCTGGCGATGAGCGAAATGTAGTGCTTACGTTGTCCCGCAT
 TTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAAGGATGTCGCTGCCGACTGGG
 CAATGGAGCGCCTGCCGGCCAGTATCAGCCCGTCATACTTGAAGCTAGACAGGCTT
 30 ATCTTGGACAAGAAGAAGATCGCTTGGCCTCGCGCGCAGATCAGTTGGAAGAATTT
 GTCCACTACGTGAAAGGCGAGATCACCAAGGTAGTgGGCAAAGaaCAAAAACCTCATT
 TCTGAAGAAGACTTGTAAGTGCAGATAACCCAAATAATGTTTTAAATTTTAAAAAT
 AATGTAGGAGGAAAAATTATGGCTAGCAGTAAAGGAGAAGAACTTTTCACTGGAGT
 TGTCCCAATTCTTGTGTAATTAGATGGTGTGTTAATGGGCACAAATTTTCTGTCAGT
 35 GGAGAGGGTGAAGGTGATGCAACATACGGAAACTTACCCTTAAATTTATTTGCACT
 ACTGGAAACTACCTGTTCCtTGCCAACACTTGTCACTACTTTCTCTTATGGTGTTC
 AATGCTTTTCAAGATACCCAGATCATATGAAGCGGCACGACTTCTTCAAGAGCGCCA

TGCCTGAGGGATACGTGCAGGAGAGGACCATCTCTTTCAAGGACGACGGGAACACTAC
 AAGACACGTGCTGAAGTCAAGTTTGAGGGAGACACCCTCGTCAACAGGATCGAGCT
 TAAGGGAATCGATTTCAAGGAGGACGGAAACATCCTCGGCCACAAGTTGGAATACA
 ACTACAACCTCCACAAACGTATACATCACGGCAGACAAACAAAAGAATGGAATCAAA
 5 GCTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGACCA
 TTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTA
 CCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGG
 TCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACA
 AATAAGctctagCTAGAGCgatectggcctagtctataggagggtttgaaaagaaggagcaataatcattttctgttctatcaag
 10 aggggtgctattgctcctttctttttctttttattttacttagtattttacttacatagactttttgtttacattatagaaaaagaaggagaggttatttt
 ctgcatattcatgGGGGATCAAAGCTT (SEQ ID NO: 1)

Transformation and Selection of Transplastomic Lines

Plastid transformation in *Arabidopsis* was carried out using our 1998 protocol, as shown
 in Fig. 3A-3F (Sikdar et al., 1998). The leaves (10 to 20 mm) were harvested from aseptically
 15 grown plants and covered the surface of agar-solidified ARMI medium in a 10 cm petri dish. We
 used ~100 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 μ m) 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-
 20 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 an additional 2 d 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,
 25 transferred onto the same medium (ARMI) containing 100 mg/L spectinomycin, incubated at
 28°C, and illuminated for 16 h with fluorescent tubes (CXL F025/741). After 8 to 10 d, 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 2 weeks until putative transplastomic clones were identified as
 30 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.

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DNA and RNA Gel-Blot Analyses

Total leaf DNA was prepared by the cetyltrimethylammonium bromide 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 were carried out as described (Kuroda and Maliga, 2001). The probes were as follows: for *aadA*, a 0.8-kb *NcoI-XbaI* fragment isolated from plasmid pHCI (Carrer et al., 1991); and for *gfp*, a fragment amplified from the *gfp* coding region using primers *gfp*-forward p1 (5'-TTTCTGTCAGTGGAGAGGGTG-3') (SEQ ID NO: 2) and *gfp*-reverse p2 (5'-CCCAGCAGCTGTTACAAACT-3') (SEQ ID NO: 3) (Fig. 2).

Alignment of Homomeric ACCases

The alignment of homomeric ACCases in the Brassicaceae family was carried out with MultAlin software (Corpet, 1988).

Accession Numbers

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

The following examples are provided to illustrate certain embodiments of the invention. They are not intended to limit the invention in any way.

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Example I

Dicistronic pATV1 Vector for 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. 2). 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. 2). 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 and Identification of Transplastomic Events

Plastid transformation was carried out in the Col-0 (Columbia) accession and the Columbia *ACC2* T-DNA insertion line *acc2-1* (SALK_148966C) shown to be sensitive to spectinomycin in the Parker et al. study (Parker et al., 2014). We also evaluated plastid transformation efficiency in the Sav-0 (Slavice) accession that was the most sensitive to spectinomycin in the study (Parker et al., 2014). The Sav-0 *ACC2* gene carries 15 missense mutations, but one variant alone (G135E) that alters a conserved residue immediately preceding the biotin carboxylase domain appears to be responsible for the hypersensitive phenotype (Parker et al., 2016). Plants were grown aseptically on ARM5 medium (Fig. 3A); leaves for plastid transformation were harvested from sterile plants and placed on ARMI media. 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 to create a series of parallel incisions 1 mm apart. The mechanical wounds are essential to induce uniform callus formation in the leaf blades. The stamped leaves were 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 (four bombarded plates), no transplastomic event was found. We obtained eight events on five bombarded plates using leaf tissue in the *acc2-1* mutant background and four events in four bombarded plates in the Sav-0 accession (Table 1). This transformation efficiency is comparable to the transformation efficiency obtained with current protocols in tobacco: four to five 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 (Sikdar et al., 1998) but has been largely unsuccessful. For example, bombardment of 26 plates of RLD and five plates of Landsberg erecta (Ler) leaf tissue did not yield a transplastomic event (Table 1). In contrast, nine bombardments of leaves with the *acc2* null background yielded 12 transplastomic clones. Even though the technology improved significantly 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*.

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.

Plasmid	Left/Right Arm	Marker Gene	Accession	Tissue	Gun	No. of Plates	No. of Transplastomic Events	Reference
<i>kb</i>								
pGS31A	1.1/0.9	Prrn:trbcl:aadA:TpsbA	RLD	Leaf	Single, Tu/1 μ m	201	2	Sikdar et al. (1998)
pAAK176	1.7/0.8	Prrn:trbcl:aadA:TpsbA	RLD	Leaf	Hepta, Au/0.6 μ m	10	0	Reported here
			Ler	Leaf	Hepta, Au/0.6 μ m	4	0	Reported here
pTT626	1.7/0.8	Prrn:Lcry9:aadA-gfp:TpsbA	RLD	Leaf	Hepta, Au/0.6 μ m	14	0	Reported here
pATV1	1.7/0.8	Prrn:LapB:aadA:Lcry9-gfp:TpsbA	RLD	Leaf	Hepta, Au/1 μ m	2	0	Reported here
			Ler	Leaf	Hepta, Au/1 μ m	1	0	Reported here
			Col-0	Leaf	Hepta, Au/0.6 μ m	4	0	Reported here
			Col-0 <i>acc2-1</i>	Leaf	Hepta, Au/0.6 μ m	5	8	Reported here
			Sav-0	Leaf	Hepta, Au/0.6 μ m	4	4	Reported here

Confocal Microscopy to Confirm Transplastomic events

Because GFP is encoded in the second ORF, GFP accumulation is expected only if the mRNA is translated in plastids on the prokaryotic type 60S ribosomes known to translate transgenic polycistronic mRNAs. Examples are the plastid *psbE* operon (Carrillo et al., 1986; Willey and Gray, 1989), the *psaA/B* transcript (Meng et al., 1988) and *petA*, which is not cleaved

off the upstream *ycf10* gene (Willey and Gray, 1990). Translation of polycistronic mRNAs created by operon extension has also been demonstrated (Staub and Maliga, 1995). Thus, GFP accumulation was anticipated only if the *gfp* gene is expressed in chloroplasts.

The putative transplastomic lines identified as green cell clusters have subsequently been confirmed as transplastomic events by detecting localization of GFP to plastids by confocal microscopy. 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 the uniform accumulation of GFP in the leaves of a Sav-0 #6 plant shown in Fig. 4 and by DNA gel-blot analyses of calli shown in Fig. 5B.

Regeneration of Transplastomic Sav-0 Plants and Transmission of GFP to Seed Progeny

After the bombardment of Col-0 and Sav-0 leaves, the 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, the calli did not proliferate. Therefore, we transferred the transplastomic calli to media that were used successfully 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 d) exposed to callus induction medium containing 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.05 mg/L kinetin and then transferred to a shoot regeneration medium containing 0.15 mg/L indole acetic acid (IAA) and 1.6 mg/L phenyladenine. Phenyladenine is a potent compound for shoot regeneration through the inhibition of cytokinin oxidase/dehydrogenase activity (Motte et al., 2013). Shoots from the calli

developed in 45 to 60 days and flowered and formed siliques in sterile culture (Fig. 3E). The plants glowed intensely when illuminated with UV light, indicating high-level GFP accumulation (Fig. 3F-3G). Confocal microscopy suggests uniform transformation of plastid genomes in the leaves of Sav-0 #6 plants (Fig. 4) and was confirmed by molecular analyses (Fig. 5B).

The transplastomic shoots were transferred to larger 500-mL Erlenmeyer flasks containing ARM for seed set, where they continued to grow.

Molecular Analysis of Transplastomic Arabidopsis Clones

DNA and RNA gel blot analyses was carried out on the callus and shoots of the two 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 were carried out, confirming uniform transformation of the plastid genomes in both calli and shoots (Fig. 5A). RNA gel blot analyses indicated the presence of a 2-kb dicistronic transcript detected by both the *aadA* and *gfp* probes (Fig. 5B).

DISCUSSION

Development of successful plastid transformation protocols takes multiple years, explaining the relative paucity of crops in which plastid transformation is routine (Maliga and Bock, 2011; Maliga, 2012; Bock, 2015). The expectation is to obtain transplastomic plants, which carry and transmit to the seed progeny a uniformly transformed plastid genome population. The time required to obtain a flowering transplastomic plants from seed takes about 5 to 6 months, as outlined in Table 2. This time frame can be broken up into discrete steps, each of which represents a milestone in developing a complete system. We report here a significant break-through: high frequency transformation of the Arabidopsis plastid genome in spectinomycin sensitive accessions and a marker system that enables rapid identification of transplastomic events by selective expression of a GFP gene in plastids. This step is a major advance towards developing a complete system of plastid engineering in Arabidopsis.

Table 2. An overview of the protocol for the construction of a transplastomic Arabidopsis Sav-0 plants.

	OBJECTIVE	CULTURE MEDIUM	TIME
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			(No. of transfers)
Step 1	Seed germination	ARM5 Medium	14 days
Step 2	Grow sterile plants	ARM5 medium	42 days
Step 3	Leaf callus, non selective	ARMI medium	4 days
Step 4	Leaf bombardment	ARMI medium	
Step 5	Leaf callus, non selective	ARMI medium	2 days
Step 6	Leaf callus, selective	ARMI medium + Spectinomycin (100 mg/L)	14 days
Step 7	Leaf callus, greening	ARMIIr + Spectinomycin (100 mg/L)	21 days (2x)
Step 8	Shoot induction	ARM medium + 2,4-D (0.5 mg/L), kinetin (0.05 mg/L), Spectinomycin (100 mg/L)	3 days
Step 9	Shoot regeneration	ARM medium + IAA (0.15 mg/L), Phe-Ade (1.6 mg/L), Spectinomycin (100 mg/L)	45-60 days

Time to flowering plants: **145 - 160 days (~5 months)**

Development of a Plastid Transformation Protocol in Arabidopsis

The steps of a complete system of plastid engineering in Arabidopsis consist of: (a) obtaining or generating sterile *acc2* defective plants to provide a leaf source for transformation; (b) delivering DNA to plastids; (c) recovering transplastomic events; (d) regenerating shoots from transplastomic callus and (e) obtaining seed from the shoots.

We report here approximately 100-fold enhanced plastid transformation efficiency per bombardment in the *acc2* null background: eight events in five bombarded samples in the Col-0 *acc2-1* line and four events in four bombarded samples in the Sav-0 background. The increase from one event per approximately 100 bombardments to one event per one bombardment is due in part 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). The 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. The use of the *acc2* knockout lines shortened scoring because the proliferation of non-transformed cells growth was efficiently inhibited by spectinomycin, enabling identification of the spectinomycin-resistant green cell clusters. Spectinomycin resistance may be due to the integration of *aadA* in the plastid 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 chloroplasts, enabling the 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. Col-0 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. Shoot regeneration protocols have been worked out from root (Marton and Browse, 1991) and leaf explants (Lutz et al., 2015) of the RLD ecotype; 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) ecotype. Thus, a routine protocol for plastid transformation in *Arabidopsis* can be obtained by the refinement of leaf regeneration protocol in the Sav-0 ecotype, or by developing *ACC2* knockout mutations in the RLD (Marton and Browse, 1991) or *Wassilewskya* (Ws) (Chupeau et al., 2013; Zhao et al., 2014) nuclear backgrounds. Alternatively, the Col-0 *acc2-1* can be transformed with the steroid-inducible *BABYBOOM* gene to facilitate plant regeneration from transplastomic events (Lutz et al., 2015).

Seed from transplastomic tobacco is obtained by rooting shoots in tissue culture, then transferring the rooted cuttings to a greenhouse. *Arabidopsis* shoots obtained in tissue culture are notoriously difficult to root. Rather than making an effort to root the plants in culture and transfer them to the greenhouse, we obtained seed from plants in sterile culture, a two-three month process (Lutz et al., 2015).

Early Identification of Plastid Transformants

The dicistronic marker system is a developer's tool that enables early scoring, but severely burdens the developing plants due to the high level of AAD and GFP expression, ~7% and ~15% of total soluble cellular protein (TSP) in tobacco, respectively (unpublished). High-levels of AAD are not necessary to obtain transplastomic plants. We have found that a mutation
5 in the promoter of the *aadA* gene reduced accumulation of AAD gene product below 1% without impact on the frequency of transplastomic events by spectinomycin selection (Sinagawa-Garcia et al., 2009). Therefore, the new Arabidopsis vectors expressing low levels of AAD described herein can be used to advantage as lowered expression levels of AAD do not compromise plant growth.

Plastid Transformation in Arabidopsis Provides Template for Recalcitrant Crops

The recognition that the duplicated ACCase in Arabidopsis is an impediment to plastid transformation provides the guidance necessary for implementation of plastid transformation in all Arabidopsis accessions and in crops having a plastid-encoded *accD* gene and a plastid-
15 targeted ACC2 enzyme. The *Arabidopsis thaliana* ACC2 enzyme has an N-terminal extension compared to ACC1 (Fig. 6A). The N-terminal extension is a plastid targeting sequence shown by subcellular localization of a GFP fusion protein (Babychuk 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
20 ACC2 enzyme in these species has an N-terminal extension compared to ACC1 (Fig. 6B and 6C). Thus, a targeted mutation in the N-terminal extension can selectively inactivate the ACC2 variant to create a spectinomycin hypersensitive variant similar to the Col-0 *acc2-1* deletion derivative (Parker et al., 2014).

Crops recalcitrant to plastid transformation such as cotton (*Gossypium raimondii*),
25 soybean (*Glycine max*) and alfalfa (*Medicago truncatula*) have a plastid *accD* gene and multiple homomeric nuclear ACC genes. Indeed, this method should prove effective in those plants having comparable *ACC2* with an N-terminal extension. Moreover, further experimentation could be performed to determine how deletion of one or more of the homomeric ACCase genes enhances recovery of transplastomic events.

30 Mutations in genes other than *ACC2* also made Arabidopsis sensitive to spectinomycin. The TIC20-IV gene, which is required for the import of proteins through the inner chloroplast

membrane, appears to limit the import of ACC2 enzyme (Parker et al., 2014). Dicot plastid genomes have several essential genes, including *accD*, *clpP*, *Ycf1* and *Ycf2* (Scharff and Bock, 2014). Apparently, in photoheterotrophic cultures where sucrose in the medium eliminates the need for photosynthesis, only translation of the *accD* mRNA, hence fatty acid biosynthesis, is required to sustain plant life.

Conclusion

Boost of plastid transformation efficiency using ACC2 knockout lines in commercial species of Brassicaceae has obvious economic benefits. Genomic resources make Arabidopsis the favored model to study basic biological processes, and to explore new biotechnological applications (Weigel and Mott, 2009; Koornneef and Meinke, 2010; Stitt et al., 2010; Wallis and Browse, 2010). The exception is photosynthesis research and chloroplast biotechnology that utilizes tobacco (*Nicotiana tabacum*) because engineering of the plastid genome encoding key components of the photosynthetic machinery is routine in only this species (Hanson et al., 2016; Sharwood et al., 2016). If plastid transformation would be available in Arabidopsis, this research would be carried out in this model organism, in which a large mutant collection is available in virtually any nuclear gene contributing to photosynthesis. Recognizing the importance of plastid translation during selection of transplastomic events has identified a bottleneck of plastid transformation in *Arabidopsis*. High frequency plastid transformation in *Arabidopsis thaliana* will open up the unique resources of this model species to advance our understanding of plastid function and new biotechnological applications.

Example II

Deletion of *ACC2* genes in Brassicaceae crops to create suitable recipients for plastid transformation

As discussed above in Example I, crops in the Brassicaceae family encode homologs of the Arabidopsis *ACC2* gene, characterized by an N-terminal extension as compared to *ACC1*. Manual inspection of the N-terminal region of *ACC2* genes led to the identification of >20 suitable guide RNAs (see Table 3). The potential gRNAs targeting both strands (5' to 3' and 3' to 5') are identified as NNNNNNNNNNNNNNNNNNNN NGG sequence (20N+NGG, N=A/G/C/T) (SEQ ID NO: 4), where the only limitation is the presence of a GG sequence (Mali

et al., 2013). More relaxed rules for sgRNA design can be used in plants, such as G(N)₁₉₋₂₂ for the U6 promoter and A(N)₁₉₋₂₂ for the U3 promoter and the 1st nucleotide does not have to match the genomic sequence (Belhaj et al., 2013).

Brassica napus L. (AACC, 2n=4x=38) is an amphidiploid species originating from spontaneous hybridization of *Brassica rapa* (AA, 2n=2x=20) and *Brassica oleracea* (CC, 2n=2x=18) (Song and Osborn, 1992; Howell et al., 2008). The *Brassica napus* genome encodes two *ACC1* genes (Locus106413885; Locus106418889) and two *ACC2* genes (GenBank accession numbers X77576, Y10302) (Schulte et al., 1997). Simultaneous mutation of two genomic sequences can be executed efficiently using CRISPR/Cas9, as described in the literature. A noteworthy example is simultaneous inactivation of 62 copies of a porcine endogenous retrovirus in pigs (Yang et al., 2015). Additionally, non-segregating seed progeny due to mutations in both genomic copies in the first generation of Arabidopsis and tomato plants (Feng et al., 2014) (Brooks et al., 2014). The alignment of 298 N-terminal nucleotides of the *Brassica napus* *ACC2* genes reveals 7 mismatches. Still, 9 of the 15 potential forward sgRNAs are useful for simultaneously inducing mutations in both *ACC2* gene copies (Fig. 7, Table 3).

To achieve targeted deletion in the *ACC2* N-terminal region, the gRNAs are cloned into the CRISPR/Cas vector and introduced into different crops using a nuclear transformation system appropriate for the target species. For example, *Camelina sativa* plants will be transformed by the flower dip protocol (Liu et al., 2012). In the case of *Brassica*, introduction of the CRISPR/Cas vector system can be achieved using Agrobacterium-mediated transformation of hypocotyls (Cardoza and Stewart, 2003, 2006) or flower dip transformation (Tan et al., 2011; Verma et al., 2008) as described below.

Agrobacterium-mediated transformation of hypocotyl segments

Brassica napus L. cv. Westar is transformed with an Agrobacterium binary vector carrying kanamycin resistance as a plant marker. Seeds are surface-sterilized with 10% sodium hypochlorite with 0.1% Tween for 5 minutes, followed by a 1-min rinse with 95% ethanol and washing the seed 5x with sterile distilled water. The seeds are germinated in sterile culture on MS basal medium (Murashige and Skoog, 1962) containing 20 g/l sucrose and solidified with 2 g/l Gelrite. Hypocotyls for transformation are excised from 8 to 10-day-old seedlings and 1-cm pieces preconditioned for 48 h on MS medium supplemented with 1 mg/l 2,4-D (2,4-

dichlorophenoxy acetic acid) and 30 g/l sucrose, solidified with 2 g/l Gelrite. The preconditioned hypocotyl segments were then inoculated with *Agrobacterium* grown overnight to an OD₆₀₀=0.8 in liquid LB medium. The *Agrobacterium* cells are pelleted by centrifugation and re-suspended in liquid callus induction medium with 0.05 mM acetosyringone to induce T-DNA transfer.

5 Co-cultivation with *Agrobacterium* is performed for 48 h on MS medium with 1 mg/l 2,4-D. Following co-cultivation, the explants are transferred to the same medium with 400 mg/l timentin and 200 mg/l kanamycin to select for transformed cells. After 2 weeks, the explants are transferred to MS medium to promote organogenesis containing 4 mg/l BAP (6-benzylaminopurine), 2 mg/l zeatin, 5 mg/l silver nitrate, 400 mg/l timentin, 200 mg/l kanamycin
10 and 30 g/l sucrose, solidified with 2 g/l Gelrite. After an additional 2 weeks, the tissue is transferred to MS medium containing 3 mg/l BAP, 2 mg/l zeatin, the same antibiotics, 30 g/l sucrose and 2 g/l Gelrite for shoot development. To encourage shoot elongation, the shoots are transferred to MS medium with 0.05 mg/l BAP, 30 g/l sucrose, antibiotics as above, solidified with 3 g/l Gelrite. The elongated shoots are rooted on a medium containing half-strength MS
15 salts, 10 mg/l sucrose, 3 g/l Gelrite, 5 mg/l IBA, and 400 mg/l timentin and 200 mg/l kanamycin. The cultures are incubated at 25±2°C, 16/8-h (light/dark) photoperiod. The rooted shoots are transferred to soil and grown at 20°C 20, 16/8 h (light/dark) photoperiod. To prevent desiccation, the plants are initially covered with a plastic dome.

20 **Floral dip transformation in *Brassica* spp. to generate ACC2 defective plants**

For *Agrobacterium*-mediated floral dip transformation of *Brassica napus*, for example cv. Westar, more recent protocols that do not require vacuum infiltration are preferred. Verma et al. (2008) and Tan et al. (2011) report such protocols. Verma et al. (2008) recommends growing up the *Agrobacterium* strain in a selective medium, harvesting the cells by centrifugation and then
25 re-suspending them in transformation medium comprising half MS salts, 5% sucrose, 0.05% Silwet L-77 to obtain the desired density (OD₆₀₀= 0.8 to 2.0). Plants are inoculated by submerging inflorescences in the bacterial suspension for one minute and then the inflorescences are wrapped with Saran wrap for 24 h to maintain the humidity. Seeds are collected at maturity and germinated on a selective medium to identify T1 seedlings by the expression plant marker
30 encoded in the T-DNA.

A variant of this protocol is described by (Tan et al., 2011). *Agrobacterium* cultures carrying a target construct are collected by centrifugation and then resuspended in a solution containing 0.53 MS salts, 3% Sucrose, 0.1% Silwet L-77, 2 mg/L 6-benzyladenine, and 8 mg/L acetosyringone. The inflorescence of flowering plants is dipped into a beaker containing the *Agrobacterium* culture for 1 to 2 min with gentle agitation, and the treated inflorescence is wrapped with Saran wrap to keep the flowers moist. The plants are treated three times at two day intervals, then the plants are allowed to grow to maturation. Seeds harvested from the transformed plants were surface sterilized and sown on the MS medium containing the plant marker encoded in the T-DNA. If kanamycin resistance is the plant marker, 200 mg/L kanamycin is used to screen for putative transformants. The putative transformants are identified upon the initiation of the first pair of green true leaves. Additional protocols for floral dip transformation are listed in the Table below.

Species	Reference
<i>Brassica rapa</i> L. ssp <i>chinensis</i>	(Qing et al., 2000)
<i>Brassica campestris</i> L. ssp <i>chinensis</i>	(Liu et al., 1998)
<i>B. napus</i>	(Wang et al., 2003; Wang et al., 2005; Tan et al., 2011)
<i>B. napus</i> , <i>B. carinata</i> , high freq.	(Verma et al., 2008)
<i>Camelina sativa</i>	(Lu and Kang, 2008)

There are several *Agrobacterium* vector systems that have been described for CRISPR/Cas mutagenesis in plants (Belhaj et al., 2013; Li et al., 2014). We prefer the system described by Mao et al. for its simplicity (Mao et al., 2013). When a population of homozygous *ACC2* knockout or biallelic mutant population is obtained, the seeds will be germinated on spectinomycin medium to identify the *ACC2* defective plants by spectinomycin sensitivity (Parker et al., 2014). The type of knockout mutation will be verified by sequencing the target region and the progeny will be used as recipient in chloroplast transformation experiments. *Brassica juncea* is also an oilseed crop. The genomics of this crop is relatively undeveloped. However, guide RNAs to knockout the *ACC2* gene can be designed using the principles outlined for the other Brassicaceae species as described herein above.

Table 3. Identification of guide RNAs in the N-terminal extension of the *ACC2* gene in Brassicaceae species. Shown are the N-terminal protein sequence, the corresponding cDNA sequence and the potential gRNAs.

5 >*A. thaliana* ACC2 (At1g36180)

>MEMRALGSSCSTGNNGGSAPITLTNISPWITTVFPSTVKLRSSLRTFKGVSSRVRTFKGVSTRVLSRTKQQFPLFCFLNPDPISFLENDVSEAERTVVL (SEQ ID NO: 5)

10 >ATGGAGATGAGAGCTTTGGGTTCTTCGTGTTCTACTGGTAATGGAGGTTCTGCTCC
GATTACCCTCACGAATATATCTCCATGGATCACAACAGTTTTTCCGTCGACAGTGAA
GCTGAGAAGTAGTTTGAGAACCTTCAAAGGAGTTTCGTCAAGAGTGAGAACCTTTA
AAGGAGTTTCTTCGACAAGAGTTTTGTCTCGGACCAAACAACAGTTTCCTCTGTTTTG
TTTCCTAAACCCTGATCCGATCTCCTTCTTGGAATGATGTATCTGAAGCTGAAAG
15 GACAGTAGTTTTACCG (SEQ ID NO: 6)

For potential gRNAs, see SEQ ID NO. 239-254 below.

> *A. lyrata* ACC2 (XM_GG2891167.1)

20 >MEMRALVSSCATGNNGGSDPFSFTKVSPWITTVGGKDRDFPTTVKLRTSMRTFKGV
SIRGRTFKGVSTRVLSRNKQQFPLFCFLNPDPSTFRDNDISEAQR (SEQ ID NO: 7)

>5'-3'

25 ATGGAGATGAGAGCTTTGGTTTCTTCGTGTGCTACCGGTAATGGAGGTTCTGATCCG
TTTAGCTTCACGAAAGTTTCTCCATGGATCACAACAGTTGGTGGTAAGGACAGAGAT
TTTCCAACGACAGTGAAGCTAAGAACTAGTATGAGAACCTTTAAAGGAGTTTCTATA
AGAGGGAGAACCTTTAAAGGAGTTTCGACAAGAGTTTTGTCTCGGAACAAACA
GTTTCCTCTGTTTTGTTTCCTAAACCCTGATCCGACCTCCTTCCGGGATAATGATATA
30 TCTGAAGCTCAAAGG (SEQ ID NO: 8)

- TTGGTTTCTTCGTGTGCTAC CGG (SEQ ID NO: 9)
TCTTCGTGTGCTACCGGTAA TGG (SEQ ID NO: 10)
TCGTGTGCTACCGGTAATGG AGG (SEQ ID NO: 11)
GCTTCACGAAAGTTTCTCCA TGG (SEQ ID NO: 12)
5 TCTCCATGGATCACAACAGT TGG (SEQ ID NO: 13)
CCATGGATCACAACAGTTGG TGG (SEQ ID NO: 14)
GATCACAACAGTTGGTGGTA AGG (SEQ ID NO: 15)
ACTAGTATGAGAACCTTTAA AGG (SEQ ID NO: 16)
TTTAAAGGAGTTTCTATAAG AGG (SEQ ID NO: 17)
10 ATAAGAGGGAGAACCTTTAA AGG (SEQ ID NO: 18)
TTTCGACAAGAGTTTTGTCT CGG (SEQ ID NO: 19)
ACCCTGATCCGACCTCCTTC CGG (SEQ ID NO: 20)
ATGATATATCTGAAGCTCAA AGG (SEQ ID NO: 21)
- 15 >3'-5'
CCTTTGAGCTTCAGATATATCATTATCCCGGAAGGAGGTCGGATCAGGGTTTAGGAA
ACAAAACAGAGGAAACTGTTGTTTGTTCGAGACAAAACCTCTTGTCGAAACTCCTTT
AAAGGTTCTCCCTCTTATAGAAACTCCTTTAAAGGTTCTCATACTAGTTCTTAGCTTC
ACTGTCGTTGGAAAATCTCTGTCCTTACCACCAACTGTTGTGATCCATGGAGAAACT
20 TTCGTGAAGCTAAACGGATCAGAACCTCCATTACCGGTAGCACACGAAGAAACCAA
AGCTCTCATCTCCAT (SEQ ID NO: 22)
- CTTCAGATATATCATTATCC CGG (SEQ ID NO: 23)
AGATATATCATTATCCCGGA AGG (SEQ ID NO: 24)
25 TATATCATTATCCCGGAAGG AGG (SEQ ID NO: 25)
TCATTATCCCGGAAGGAGGT CGG (SEQ ID NO: 26)
TCCCGGAAGGAGGTCGGATC AGG (SEQ ID NO: 27)
CCCGGAAGGAGGTCGGATCA GGG (SEQ ID NO: 28)
AGGAGGTCGGATCAGGGTTT AGG (SEQ ID NO: 29)
30 GGGTTTAGGAAACAAAACAG AGG (SEQ ID NO: 30)
TCTTGTCGAAACTCCTTTAA AGG (SEQ ID NO: 31)

TCTTATAGAAACTCCTTTAA AGG (SEQ ID NO: 32)

GTTCTTAGCTTCACTGTCGT TGG (SEQ ID NO: 33)

CCACCAACTGTTGTGATCCA TGG (SEQ ID NO: 34)

GAAACTTTCGTGAAGCTAAA CGG (SEQ ID NO: 35)

5 CGGATCAGAACCTCCATTAC CGG (SEQ ID NO: 36)

>*C. sativa* ACC2-1(LOC104777495)

>MEMRALVSSYSTGNNGSDPISLTNGSPWITTVGGGASTMDREFPLTVKLGSSMRAFKG

10 VSTTTVLSRTKQQFPLVCLARNNANSTDPTSFWENDISEVQR (SEQ ID NO: 37)

>5'-3'

ATGGAGATGAGAGCTTTGGTTTCTTCGTATTCTACCGGTAATGGAGGTTCTGATCCG

ATCAGCCTCACGAATGGTTCTCCATGGATCACAACAGTTGGTGGTGGTGCAAGTACC

15 ATGGACAGAGAGTTTCCATTGACTGTGAAGCTGGGAAGTAGTATGAGAGCCTTCAA

AGGAGTAAGCACACAACAGTTTTGTCTCGGACCAAACAACAGTTTCCTCTGGTATG

CTTAGCAAGAAACAATGCGAACAGCACTGATCCGACCTCGTTCTGGGAGAATGATA

TATCTGAAGTTCAAAGG (SEQ ID NO: 38)

20 TTGGTTTCTTCGTATTCTAC CGG (SEQ ID NO: 39)

TCTTCGTATTCTACCGGTAA TGG (SEQ ID NO: 40)

TCGTATTCTACCGGTAATGG AGG (SEQ ID NO: 41)

GATCCGATCAGCCTCACGAA TGG (SEQ ID NO: 42)

GCCTCACGAATGGTTCTCCA TGG (SEQ ID NO: 43)

25 TCTCCATGGATCACAACAGT TGG (SEQ ID NO: 44)

CCATGGATCACAACAGTTGG TGG (SEQ ID NO: 45)

TGGATCACAACAGTTGGTGG TGG (SEQ ID NO: 46)

TGGTGGTGGTGCAAGTACCA TGG (SEQ ID NO: 47)

GTTTCCATTGACTGTGAAGC TGG (SEQ ID NO: 48)

30 AGTAGTATGAGAGCCTTCAA AGG (SEQ ID NO: 49)

GCACAACAACAGTTTTGTCT CGG (SEQ ID NO: 50)

GACCAAACAACAGTTTCCTC TGG (SEQ ID NO: 51)

GCACTGATCCGACCTCGTTC TGG (SEQ ID NO: 52)

ATGATATATCTGAAGTTCAA AGG (SEQ ID NO: 53)

5 >3'-5'

CCTTTGAACTTCAGATATATCATTCTCCCAGAACGAGGTCGGATCAGTGCTGTTCGC
ATTGTTTCTTGCTAAGCATACCAGAGGAACTGTTGTTTGGTCCGAGACAAAACGTG
TGTTGTGCTTACTCCTTTGAAGGCTCTCATACTACTTCCCAGCTTCACAGTCAATGGA
AACTCTCTGTCCATGGTACTTGCACCACCACCAACTGTTGTGATCCATGGAGAACCA
10 TTCGTGAGGCTGATCGGATCAGAACCTCCATTACCGGTAGAATACGAAGAAACCAA
AGCTCTCATCTCCAT (SEQ ID NO: 54)

TATATCATTCTCCCAGAACG AGG (SEQ ID NO: 55)

TCATTCTCCCAGAACGAGGT CGG (SEQ ID NO: 56)

15 TTTCTTGCTAAGCATACCAG AGG (SEQ ID NO: 57)

TACCAGAGGAACTGTTGTT TGG (SEQ ID NO: 58)

TGTTGTGCTTACTCCTTTGA AGG (SEQ ID NO: 59)

CTTCCCAGCTTCACAGTCAA TGG (SEQ ID NO: 60)

CAATGGAACTCTCTGTCCA TGG (SEQ ID NO: 61)

20 CCACCAACTGTTGTGATCCA TGG (SEQ ID NO: 62)

TCCATGGAGAACCATTCTGTG AGG (SEQ ID NO: 63)

GAACCATTCTGTGAGGCTGAT CGG (SEQ ID NO: 64)

CGGATCAGAACCTCCATTAC CGG (SEQ ID NO: 65)

25 >*C. sativa* ACC2-2 (LOC104742086) variant1

>MEMRALVSSCSTGNGGSDPISLTNGSPWITTVGGGASTMDREFPATVKLGSSMRAFKG
VSTITVLSRTKQQFPLVCLARNNGNSTDPTSFWENDISETQR (SEQ ID NO: 66)

30 >5'-3'

ATGGAGATGAGAGCTTTGGTTTCTTCGTGTTCTACGGGGAATGGAGGGTCTGATCCG
 ATCAGCCTCACGAATGGTTCTCCATGGATCACAAACAGTTGGTGGTGGTGCAAGTACC
 ATGGACAGAGAGTTTCCAGCGACTGTGAAGCTGGGAAGTAGTATGAGAGCCTTCAA
 AGGAGTAAGCACATAACAGTTCTGTCTCGGACCAAACAACAGTTTCCTCTGGTATG
 5 CTTAGCAAGAAACAACGGAAACAGCACTGATCCGACCTCGTTCTGGGAGAACGATA
 TATCTGAAACTCAAAGG (SEQ ID NO: 67)

TTTGGTTTCTTCGTGTTCTA CGG (SEQ ID NO: 68)
 TTGGTTTCTTCGTGTTCTAC GGG (SEQ ID NO: 69)
 10 TGGTTTCTTCGTGTTCTACG GGG (SEQ ID NO: 70)
 TCTTCGTGTTCTACGGGGAA TGG (SEQ ID NO: 71)
 TCGTGTTCTACGGGGAATGG AGG (SEQ ID NO: 72)
 GATCCGATCAGCCTCACGAA TGG (SEQ ID NO: 73)
 GCCTCACGAATGGTTCTCCA TGG (SEQ ID NO: 74)
 15 TCTCCATGGATCACAAACAGT TGG (SEQ ID NO: 75)
 CCATGGATCACAAACAGTTGG TGG (SEQ ID NO: 76)
 TGGATCACAAACAGTTGGTGG TGG (SEQ ID NO: 77)
 TGGTGGTGGTGCAAGTACCA TGG (SEQ ID NO: 78)
 GTTTCAGCGACTGTGAAGC TGG (SEQ ID NO: 79)
 20 AGTAGTATGAGAGCCTTCAA AGG (SEQ ID NO: 80)
 GCACAATAACAGTTCTGTCT CGG (SEQ ID NO: 81)
 GACCAAACAACAGTTTCCTC TGG (SEQ ID NO: 82)
 GTATGCTTAGCAAGAAACAA CGG (SEQ ID NO: 83)
 GCACTGATCCGACCTCGTTC TGG (SEQ ID NO: 84)
 25 ACGATATATCTGAAACTCAA AGG (SEQ ID NO: 85)

>3'-5'

CCTTTGAGTTTCAGATATATCGTTCTCCCAGAACGAGGTCGGATCAGTGCTGTTTCCG
 TTGTTTCTTGCTAAGCATAACCAGAGGAAACTGTTGTTTGGTCCGAGACAGAACTGTT
 30 ATTGTGCTTACTCCTTTGAAGGCTCTCATACTACTTCCCAGCTTCACAGTCGCTGGAA
 ACTCTCTGTCCATGGTACTTGCACCACCACCAACTGTTGTGATCCATGGAGAACCAT

TCGTGAGGCTGATCGGATCAGACCCTCCATTCCCCGTAGAACACGAAGAAACCAAA
GCTCTCATCTCCAT (SEQ ID NO: 86)

TATATCGTTCTCCCAGAACG AGG (SEQ ID NO: 87)

5 TCGTTCTCCCAGAACGAGGT CGG (SEQ ID NO: 88)

TTTCTTGCTAAGCATACCAG AGG (SEQ ID NO: 89)

TACCAGAGGAAACTGTTGTT TGG (SEQ ID NO: 90)

TATTGTGCTTACTCCTTTGA AGG (SEQ ID NO: 91)

CTTCCCAGCTTCACAGTCGC TGG (SEQ ID NO: 92)

10 CGCTGGAAACTCTCTGTCCA TGG (SEQ ID NO: 93)

CCACCAACTGTTGTGATCCA TGG (SEQ ID NO: 94)

TCCATGGAGAACCATTCTGTG AGG (SEQ ID NO: 95)

GAACCATTCTGTGAGGCTGAT CGG (SEQ ID NO: 96)

15 >**C. rubella ACC2(CARUB_v1GG08063mg)**

>MEMRALVSSCSTGNNGSDPISLTNVSPWITTVGGGASSIDREFPTTVKLGSSLRTFKGVS
STTVLSRTKQQFPLVCLARNNANSTDPTLFWENDISEAQS (SEQ ID NO: 97)

20 >5'-3'

ATGGAGATGAGAGCTTTGGTTTCTTCGTGTTCTACCGGTAATGGAGGTTCTGATCCG

ATTAGCCTCACGAATGTTTCTCCATGGATCACAACAGTTGGTGGTGGTGCAAGTTCC

ATTGACAGAGAGTTTCCAACGACTGTGAAGCTGGGAAGTAGTCTGAGAACTTTCAA

AGGAGTAAGCTCTACGACAGTTTTGTCTCGGACCAACAACAGTTTCCTCTGGTTTG

25 TTTAGCAAGAAACAATGCCAACAGCACTGATCCAACCTTGTTCTGGGAAAATGACAT
ATCTGAAGCTCAAAGC (SEQ ID NO: 98)

TTGGTTTCTTCGTGTTCTAC CGG (SEQ ID NO: 99)

TCTTCGTGTTCTACCGGTAA TGG (SEQ ID NO: 100)

30 TCGTGTTCCTACCGGTAATGG AGG (SEQ ID NO: 101)

GCCTCACGAATGTTTCTCCA TGG (SEQ ID NO: 102)

TCTCCATGGATCACAAACAGT TGG (SEQ ID NO: 103)

CCATGGATCACAAACAGTTGG TGG (SEQ ID NO: 104)

TGGATCACAAACAGTTGGTGG TGG (SEQ ID NO: 101)

GTTTCCAACGACTGTGAAGC TGG (SEQ ID NO: 105)

5 AGTAGTCTGAGAACTTTCAA AGG (SEQ ID NO: 106)

GCTCTACGACAGTTTTGTCT CGG (SEQ ID NO: 107)

GACCAAACAACAGTTTCCTC TGG (SEQ ID NO: 108)

GCACTGATCCAACCTTGTTT TGG (SEQ ID NO: 109)

10 >3'-5'

GCTTTGAGCTTCAGATATGTCATTTTCCCAGAACAAGGTTGGATCAGTGCTGTTGGC

ATTGTTTCTTGCTAAACAAACCAGAGGAACTGTTGTTTGGTCCGAGACAAAACGTG

CGTAGAGCTTACTCCTTTGAAAGTTCTCAGACTACTTCCCAGCTTCACAGTCGTTGGA

AACTCTCTGTCAATGGAACCTGCACCACCACCAACTGTTGTGATCCATGGAGAAACA

15 TTCGTGAGGCTAATCGGATCAGAACCTCCATTACCGGTAGAACACGAAGAAACCAA

AGCTCTCATCTCCAT (SEQ ID NO: 110)

TATGTCATTTTCCCAGAACA AGG (SEQ ID NO: 111)

TCATTTTCCCAGAACAAGGT TGG (SEQ ID NO: 112)

20 CAAGGTTGGATCAGTGCTGT TGG (SEQ ID NO: 113)

TTTCTTGCTAAACAAACCAG AGG (SEQ ID NO: 114)

AACCAGAGGAACTGTTGTT TGG (SEQ ID NO: 115)

CTTCCCAGCTTCACAGTCGT TGG (SEQ ID NO: 116)

CGTTGGAACTCTCTGTCAA TGG (SEQ ID NO: 117)

25 CCACCAACTGTTGTGATCCA TGG (SEQ ID NO: 118)

TCCATGGAGAAACATTCGTG AGG (SEQ ID NO: 119)

GAAACATTCGTGAGGCTAAT CGG (SEQ ID NO: 120)

CGGATCAGAACCTCCATTAC CGG (SEQ ID NO: 121)

30 >*B. oleracea* ACC2 (LOC106301042)

>MEMRALVSCSAAGNGASDRFRLSNVSPWITSARGASGSDSPATVKLRSSSMIRAFKGV
SIYKNKTRRNVLSQRNKQFRPMAYLGRKDLSSPDPTSFCND (SEQ ID NO: 122)

>5'-3'

5 ATGGAGATGAGAGCTTTGGTTTCGTGTTCTGCTGCCGGAATGGAGCTTCTGATCGG
TTAGACTCTCCAATGTTTCACCATGGATCACATCTGCTCGTGGTGCAAGTGGCAGT
GACTCCCCAGCCACAGTGAAGCTGAGAAGCAGCTCTATGATTAGAGCTTTCAAAGG
AGTTTCGATTTACAAAAACAAGACCAGAAGAAATGTTCTGTCTCAAAGGAACAAAC
AGTTCCGTCCTATGGCCTACTTAGGAAGGAAGGACTTGAGCAGCCCTGATCCGACCT
10 CCTTCTGCGATAATGAT (SEQ ID NO: 123)

TTGGTTTCGTGTTCTGCTGC CGG (SEQ ID NO: 124)

TCGTGTTCTGCTGCCGAAA TGG (SEQ ID NO: 125)

CCGGAATGGAGCTTCTGAT CGG (SEQ ID NO: 126)

15 GACTCTCCAATGTTTCACCA TGG (SEQ ID NO: 127)

CCATGGATCACATCTGCTCG TGG (SEQ ID NO: 128)

ACATCTGCTCGTGGTGCAAG TGG (SEQ ID NO: 129)

TCTATGATTAGAGCTTTCAA AGG (SEQ ID NO: 130)

GAAGAAATGTTCTGTCTCAA AGG (SEQ ID NO: 131)

20 GAACAAACAGTTCCGTCCTA TGG (SEQ ID NO: 132)

TTCCGTCCTATGGCCTACTT AGG (SEQ ID NO: 133)

GTCCTATGGCCTACTTAGGA AGG (SEQ ID NO: 134)

TATGGCCTACTTAGGAAGGA AGG (SEQ ID NO: 135)

25 3'-5'

ATCATTATCGCAGAAGGAGGTCGGATCAGGGCTGCTCAAGTCCTTCCTCCTAAGTA

GGCCATAGGACGGAAGTGTTCCTTTGAGACAGAACATTTCTTCTGGTCTTGTTT

TTGTAAATCGAACTCCTTTGAAAGCTCTAATCATAGAGCTGCTTCTCAGCTTCACTG

TGGCTGGGGAGTCACTGCCACTTGCACCACGAGCAGATGTGATCCATGGTGAAACA

30 TTGGAGAGTCTAAACCGATCAGAAGCTCCATTTCCGGCAGCAGAACACGAAACCAA

AGCTCTCATCTCCAT (SEQ ID NO: 136)

TCATTATCGCAGAAGGAGGT CGG (SEQ ID NO: 137)

TCGCAGAAGGAGGTTCGGATC AGG (SEQ ID NO: 138)

CAAGTCCTTCCTTCCTAAGT AGG (SEQ ID NO: 139)

5 TTCCTTCCTAAGTAGGCCAT AGG (SEQ ID NO: 140)

TTCCTAAGTAGGCCATAGGA CGG (SEQ ID NO: 141)

TTGAGACAGAACATTTCTTC TGG (SEQ ID NO: 143)

GCTGCTTCTCAGCTTCACTG TGG (SEQ ID NO: 144)

CTTCTCAGCTTCACTGTGGC TGG (SEQ ID NO: 145)

10 TTCTCAGCTTCACTGTGGCT GGG (SEQ ID NO: 146)

TCTCAGCTTCACTGTGGCTG GGG (SEQ ID NO: 147)

CCACGAGCAGATGTGATCCA TGG (SEQ ID NO: 148)

TGTGATCCATGGTGAAACAT TGG (SEQ ID NO: 149)

CCGATCAGAAGCTCCATTTC CGG (SEQ ID NO: 150)

15

>B. napus ACC2-1: Y10302

MEMRALVSCSAAGNGASDRFRLSNVSPWITSARGASGSDSPATVKLGSSSMIRAFKGV

IYKNKTRRNVLSQLRNKQFRPMAYLGRKDLSSPDPTSFCNDNDISEPQGTGSINGNDHSAV

RVSQVDEFCKAHGGKRPIHSILVATNGMAAVKLIRSVRAWSYQTFGSEKSISLVAMATP

20 EDMRINAEHIRIADQFMQVPGGTNNNNYANVHLIVEMAQATGVDAVWPGWGHASENP

ELPDALKAKGVIFLGPTAASMLALGDKIGSSLIAQAADVPTLPWSGSHVKIPPGSSMVTIP

EEMYRQACVYTTEEAVASCQVVGYPAMIKASWGGGGKGIREVHDDDEVRTLKQVQG

EVPGSPFIMKVASQSRHL (SEQ ID NO: 151)

>5'-3' 1ST EXON

25 ATGGAGATGAGAGCTTTAGTTTCGTGTTCTGCTGCCGGAATGGAGCTTCTGATCGG

TTTAGACTCTCCAATGTTTCACCATGGATCACATCAGCTCGTGGTGCAAGTGGCAGT

GACTCCCCAGCCACAGTGAAGCTGGGAAGCAGCTCTATGATTAGAGCTTTCAAAGG

CGTTTCGATTTACAAAAACAAGACCAGAAGGAATGTTCTGTCTCAAAGGAACAAAC

AGTTCCGTCCTATGGCCTACTTAGGAAGGAAGGACTTGAGCAGCCCTGATCCGACCT

30 CCTTCTGCGATAATG (SEQ ID NO: 152)

TTAGTTTCGTGTTCTGCTGC CGG (SEQ ID NO: 153)
 TCGTGTTCGTGCTGCCGAAA TGG (SEQ ID NO: 154)
 CCGGAAATGGAGCTTCTGAT CGG (SEQ ID NO: 155)
 5 GACTCTCCAATGTTTCACCA TGG (SEQ ID NO: 156)
 CCATGGATCACATCAGCTCG TGG (SEQ ID NO: 157)
 ACATCAGCTCGTGGTGCAAG TGG (SEQ ID NO: 158)
 CTCCCCAGCCACAGTGAAGC TGG (SEQ ID NO: 159)
 TCCCCAGCCACAGTGAAGCT GGG (SEQ ID NO: 160)
 10 TCTATGATTAGAGCTTTCAA AGG (SEQ ID NO: 161)
 TTTACAAAAACAAGACCAGA AGG (SEQ ID NO: 162)
 GAAGGAATGTTCTGTCTCAA AGG (SEQ ID NO: 163)
 GAACAAACAGTTCGTCCTA TGG (SEQ ID NO: 164)
 TTCCGTCCTATGGCCTACTT AGG (SEQ ID NO: 165)
 15 GTCCTATGGCCTACTTAGGA AGG (SEQ ID NO: 166)
 TATGGCCTACTTAGGAAGGA AGG (SEQ ID NO: 167)

>3'-5'

CATTATCGCAGAAGGAGGTCGGATCAGGGCTGCTCAAGTCCTTCCTTCCTAAGTAGG
 20 CCATAGGACGGAAGTGTTCCTTTGAGACAGAACATTCCTTCTGGTCTTGTTTTT
 GTAAATCGAAACGCCTTTGAAAGCTCTAATCATAGAGCTGCTTCCCAGCTTCACTGT
 GGCTGGGGAGTCACTGCCACTTGCACCACGAGCTGATGTGATCCATGGTGAAACATT
 GGAGAGTCTAAACCGATCAGAAGCTCCATTTCCGGCAGCAGAACACGAAACTAAAG
 CTCTCATCTCCAT (SEQ ID NO: 168)
 25 TCGCAGAAGGAGGTCGGATC AGG (SEQ ID NO: 169)
 CGCAGAAGGAGGTCGGATCA GGG (SEQ ID NO: 170)
 CAAGTCCTTCCTTCCTAAGT AGG (SEQ ID NO: 171)
 TTCCTTCCTAAGTAGGCCAT AGG (SEQ ID NO: 172)
 TTCCTAAGTAGGCCATAGGA CGG (SEQ ID NO: 173)

TTGAGACAGAACATTCCTTC TGG (SEQ ID NO: 174)

CTTCCCAGCTTCACTGTGGC TGG (SEQ ID NO: 175)

TTCCCAGCTTCACTGTGGCT GGG (SEQ ID NO: 176)

TCCCAGCTTCACTGTGGCTG GGG (SEQ ID NO: 177)

5 CCACGAGCTGATGTGATCCA TGG (SEQ ID NO: 178)

TGTGATCCATGGTGAAACAT TGG (SEQ ID NO: 179)

CCGATCAGAAGCTCCATTTC CGG (SEQ ID NO: 180)

>B.napus ACC2-2: X77576

10 MEMRALVSCSAAGNGASDRFRLSNVSPWITSARGASGSDSPATVKLGSSSMIRAFKGV
 IYKNKTRRNVL SQRNKQFRPMAYLGRKDLSSPDPTSFCNDNDISEPQGTGSINGNDHSAV
 RVSQVDEFCKAHGGKRPIHRILVATNGMAAVKFIRSVRAWSYQTFGSEKSLVAMATP
 EDMRINAEHIRIADQFMQVPGGTNNNNYANVHLIVEMAEATGVDAVWPGWGHASENP
 ELPDALKAKGVIFLGPTAASMLALGDKIGSSLIAQAADVPTLPWSGSHVKIPPGSSLVTIP
 15 EEMYRQACVYTTEEAVASCQVVGYPAMIKASWGGGGKGIRKVHDDDEVRLFKQVQ
 GEVPGSPIFIMKVASQSRHLEVQLLCDQYGNVSALHSRDCSVQRRHQKIIIEGPITVAPR
 DTVKKLEQAARRLAKSVNYVGAATVEFLYSMDTGDYFFLELNPR (SEQ ID NO: 181)

>5'-3' 1ST EXON

ATGGAGATGAGAGCTTTGGTTTCGTGTTCTGCTGCCGGAATGGAGCTTCTGATCGG
 20 TTTAGACTCTCCAATGTTTCACCATGGATCACATCAGCTCGTGGTGCAAGTGGCAGT
 GACTCCCCAGCCACAGTGAAGCTGGGAAGCAGCTCTATGATCAGAGCCTTCAAAGG
 AGTTTCGATTTACAAAAACAAGACCAGAAGAAATGTTTTGTCTCAAAGGAACAAAC
 AGTTTCGTCCTATGGCCTACTTAGGAAGGAAGGACTTGAGCAGCCCTGATCCGACCT
 CTTCTGCGATAATG (SEQ ID NO: 182)

25 TTGGTTTCGTGTTCTGCTGC CGG (SEQ ID NO: 183)

TCGTGTTCTGCTGCCGAAA TGG (SEQ ID NO: 184)

CCGGAATGGAGCTTCTGAT CGG (SEQ ID NO: 185)

GACTCTCCAATGTTTCACCA TGG (SEQ ID NO: 186)

CCATGGATCACATCAGCTCG TGG (SEQ ID NO: 187)
ACATCAGCTCGTGGTGCAAG TGG (SEQ ID NO: 188)
CTCCCCAGCCACAGTGAAGC TGG (SEQ ID NO: 189)
TCCCCAGCCACAGTGAAGCT GGG (SEQ ID NO: 190)
5 TCTATGATCAGAGCCTTCAA AGG (SEQ ID NO: 191)
GAAGAAATGTTTTGTCTCAA AGG (SEQ ID NO: 192)
GAACAAACAGTTTCGTCCTA TGG (SEQ ID NO: 193)
TTTCGTCCTATGGCCTACTT AGG (SEQ ID NO: 194)
GTCCTATGGCCTACTTAGGA AGG (SEQ ID NO: 195)
10 TATGGCCTACTTAGGAAGGA AGG (SEQ ID NO: 196)
>3'-5'
CATTATCGCAGAAGGAGGTCGGATCAGGGCTGCTCAAGTCCTTCCTTCCTAAGTAGG
CCATAGGACGAAACTGTTTGTTCCTTTGAGACAAAACATTTCTTCTGGTCTTGTTTTT
GTAAATCGAAACTCCTTTGAAGGCTCTGATCATAGAGCTGCTTCCCAGCTTCACTGT
15 GGCTGGGGAGTCACTGCCACTTGCACCACGAGCTGATGTGATCCATGGTGAAACATT
GGAGAGTCTAAACCGATCAGAAGCTCCATTTCCGGCAGCAGAACACGAAACCAAAG
CTCTCATCTCCAT (SEQ ID NO: 197)
TCGCAGAAGGAGGTCGGATC AGG (SEQ ID NO: 198)
CGCAGAAGGAGGTCGGATCA GGG (SEQ ID NO: 199)
20 CAAGTCCTTCCTTCCTAAGT AGG (SEQ ID NO: 200)
TTCCTTCCTAAGTAGGCCAT AGG (SEQ ID NO: 201)
TTGAGACAAAACATTTCTTC TGG (SEQ ID NO: 202)
GTAAATCGAAACTCCTTTGA AGG (SEQ ID NO: 203)
GCTGCTTCCCAGCTTCACTG TGG (SEQ ID NO: 204)
25 CTTCCCAGCTTCACTGTGGC TGG (SEQ ID NO: 205)
TTCCCAGCTTCACTGTGGCT GGG (SEQ ID NO: 206)
TCCCAGCTTCACTGTGGCTG GGG (SEQ ID NO: 207)

CCACGAGCTGATGTGATCCA TGG (SEQ ID NO: 208)

TGTGATCCATGGTGAAACAT TGG (SEQ ID NO: 209)

CCGATCAGAAGCTCCATTTC CGG (SEQ ID NO: 210)

5 >*B. rapa* ACC2 (LOC1038715GG)

>MEMRALVSCSAAGNGASDRFRLSNVSPWITSARGASGSDSPATVKLGSSSMIRAFKGV
SIYKNKSRRNVLSQRNKQFRPMAYLGRKDLSSPDPTSFCND (SEQ ID NO: 211)

10 >5'-3'

ATGGAGATGAGAGCTTTGGTTTCGTGTTCTGCTGCCGGAAATGGAGCTTCTGATCGG
TTTAGACTCTCCAATGTTTCACCATGGATCACATCAGCTCGTGGTGCAAGTGGCAGT
GACTCCCCAGCCACAGTGAAGCTGGGAAGCAGCTCTATGATCAGAGCCTTCAAAGG
AGTTTCGATTTACAAAAACAAGAGCAGAAGAAATGTTCTGTCTCAAAGGAACAAAC
15 AGTTTCGTCCTATGGCCTACTTAGGAAGGAAGGACTTGAGCAGCCCTGATCCGACCT
CCTTCTGCGATAATGAT (SEQ ID NO: 212)

TTGGTTTCGTGTTCTGCTGC CGG (SEQ ID NO: 213)

TCGTGTTCTGCTGCCGGAAA TGG (SED ID NO: 214)

20 CCGGAAATGGAGCTTCTGAT CGG (SED ID NO: 215)

GACTCTCCAATGTTTCACCA TGG (SED ID NO: 216)

CCATGGATCACATCAGCTCG TGG (SED ID NO: 217)

ACATCAGCTCGTGGTGCAAG TGG (SED ID NO: 218)

CTCCCCAGCCACAGTGAAGC TGG (SED ID NO: 219)

25 TCTATGATCAGAGCCTTCAA AGG (SED ID NO: 220)

GAAGAAATGTTCTGTCTCAA AGG (SED ID NO: 221)

TTTCGTCCTATGGCCTACTT AGG (SED ID NO: 223)

GTCCTATGGCCTACTTAGGA AGG (SED ID NO: 224)

TATGGCCTACTTAGGAAGGA AGG (SED ID NO: 225)

30

3'-5'

ATCATTATCGCAGAAGGAGGTCGGATCAGGGCTGCTCAAGTCCTTCCTTCCTAAGTA
 GGCCATAGGACGAAACTGTTTGTTCCTTTGAGACAGAACATTTCTTCTGCTCTTGTTT
 TTGTAAATCGAAACTCCTTTGAAGGCTCTGATCATAGAGCTGCTTCCCAGCTTCACT
 GTGGCTGGGGAGTCACTGCCACTTGCACCACGAGCTGATGTGATCCATGGTGAAAC
 5 ATTGGAGAGTCTAAACCGATCAGAAGCTCCATTTCCGGCAGCAGAACACGAAACCA
 AAGCTCTCATCTCCAT (SED ID NO: 226)

TCATTATCGCAGAAGGAGGT CGG (SED ID NO: 227)
 TCGCAGAAGGAGGTCGGATC AGG (SED ID NO: 228)
 10 CAAGTCCTTCCTTCCTAAGT AGG (SED ID NO: 229)
 TTCCTTCCTAAGTAGGCCAT AGG (SED ID NO: 230)
 GTAAATCGAAACTCCTTTGA AGG (SED ID NO: 231)
 GCTGCTTCCCAGCTTCACTG TGG (SED ID NO: 232)
 CTTCCCAGCTTCACTGTGGC TGG (SED ID NO: 233)
 15 TTCCCAGCTTCACTGTGGCT GGG (SED ID NO: 234)
 TCCCAGCTTCACTGTGGCTG GGG (SED ID NO: 235)
 CCACGAGCTGATGTGATCCA TGG (SED ID NO: 236)
 TGTGATCCATGGTGAAACAT TGG (SED ID NO: 237)
 CCGATCAGAAGCTCCATTTTC CGG (SED ID NO: 238)

20

Deletion of *ACC2* gene in regenerable RLD and Ws *Arabidopsis* ecotypes

The bacterial clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) defense system has been rapidly developed as a genome-engineering tool (Belhaj et al., 2013; Mali et al., 2013; Li et al., 2014). In this approach a small RNA guides
 25 the Cas9 nuclease to the target site. The nick is then repaired by non-homologous end joining, the process most often resulting in a one-nucleotide insertion or deletion in *Arabidopsis thaliana* (Feng et al., 2014). Because our objective is knocking out the *ACC2* gene, we used the same system, an *Agrobacterium* binary transformation vector in which the sgRNA is transcribed under the control of *Arabidopsis* U6 snoRNA promoter (pAtU6) and Cas9 is expressed from the
 30 *Arabidopsis* ubiquitin promoter (pAtUBQ1) (Mao et al., 2013).

The 16 guide strands provided below are suitable for this approach.

sequence		
TCCATGGAGATATATTCGTG	AGG	(SEQ ID NO: 239)
CCCTCACGAATATATCTCCA	TGG	(SEQ ID NO: 240)
GATATATTCGTGAGGGTAAT	TGG	(SEQ ID NO: 241)
CTTCTCAGCTTCACTGTCTGA	CGG	(SEQ ID NO: 242)
CCATGGAGATATATTCGTGA	GGG	(SEQ ID NO: 243)
CTTCGACAAGAGTTTTGTCT	CGG	(SEQ ID NO: 244)
TCAAGAGTGAGAACCCTTTAA	AGG	(SEQ ID NO: 245)
TCTTCGTGTTCTACTGGTAA	TGG	(SEQ ID NO: 246)
TTGGGTTCTTCGTGTTCTAC	TGG	(SEQ ID NO: 247)
TGTCGAAGAACTCCTTTAA	AGG	(SEQ ID NO: 248)
TCTTGACGAACTCCTTTGA	AGG	(SEQ ID NO: 249)
AGTAGTTTGAGAACCCTTCAA	AGG	(SEQ ID NO: 250)
TCGTGTTCTACTGGTAATGG	AGG	(SEQ ID NO: 251)
GGAAAACTGTTGTGATCCA	TGG	(SEQ ID NO: 252)
AAACAGAGGAACTGTTGTT	TGG	(SEQ ID NO: 253)
GGGTTTAGGAAACAAAACAG	AGG	(SEQ ID NO: 254)

The target for mutagenesis was exon-1 of the *ACC2* coding region encoding the chloroplast transit peptide. This N-terminal extension is absent in the *ACC1* gene, which targets its product to the cytoplasm. To design the targeting region of the guide RNA, 240 nucleotides of *ACC2* exon-1 were pasted into the guide RNA design at the MIT Optimized CRISPR Design website. The RLD and Ws sequence has a one-nucleotide (A instead of G) mismatch compared to Columbia, a sequence variation that was considered when designing the sgRNA. The closest off target site in the Arabidopsis genome has three mismatches with this target site.

To target the *ACC2* sequence CCCTCACGAATATATCTCCATGG (2nd target site in the list; SEQ ID NO: 240), we cloned two annealed oligonucleotides that form the target site in *Bbs*I-digested CRISPR/Cas9 cassette psgR-Cas9-At (Mao *et al.*, 2013). The oligonucleotides were gattgCCTCACGAATATATCTCCA (SEQ ID NO: 255), and aaacTGGAGATATATTCGTGAGGc (SEQ ID NO: 256). The CRISPR/Cas9 cassette was then cloned in a pCambia2300 Agrobacterium binary vector and introduced into Arabidopsis by the

flower dip protocol (Clough and Bent, 1998). Plants transformed with the CRISPR/Cas9 construct were selected by germinating seeds on kanamycin medium (100 mg/L).

Kanamycin resistant seedlings (T1 generation) were screened for a mutant *ACC2* target site by the T7 exonuclease I (T7E1) assay (Xie and Yang, 2013). The T7 endonuclease recognizes and cleaves non-perfectly matched DNA. The *ACC2* target region was PCR amplified using forward primer 5'-TCTCTTCCTCCTTAAAAAGCCACA-3' (SEQ ID NO: 257) and reverse primer 5'-CTAGGATTCGAAACCAGCGT-3' (SEQ ID NO: 258) using total cellular DNA as template, the amplicons were denatured, reannealed and treated with T7E1. Mismatch caused by CRISPR/Cas9 mutagenesis resulted in T7E1 cleaving the mismatched DNA, that was visualized by gel electrophoresis.

Plants carrying mutations in *ACC2* gene copies were identified by T7E1 screening the heterozygous T1 seed progeny. Mutations in *ACC2* genes were identified in the T2 generation by sequencing PCR amplicons (Fig. 8). The *acc2* knockout mutants, in contrast to wild type, do not develop shoot meristem outgrowths when germinated on spectinomycin (Parker et al., 2014). Therefore, we collected seed from the T1 plants, and germinated a small sample on spectinomycin medium to identify non-segregating *acc2* knockout populations by spectinomycin sensitivity. An example for seedling spectinomycin hypersensitive reaction is shown in Fig. 9. Note development of primary leaves on the seedlings of the parental Ws line, and the absence of any shoot meristem outgrowth on the hypersensitive Ws-2-22 mutant (Parker et al., 2014). Following this protocol uniform, non-segregating RLD and Ws seed was obtained. Such spectinomycin hypersensitive plants are the suitable recipients for plastid transformation.

Example III

Expression of heterologous genes in *ACC2*-defective *Brassica* spp.

Reproducible, high-frequency plastid transformation in the *Brassicaceae* oilseed and vegetable crops enables plastid genome engineering in spectinomycin hypersensitive *Brassica* spp. for a variety of biotechnological applications.

One application is replacement of part or the entire plastid genome with synthetic DNA. For example, the efficiency of sunlight to biomass conversion can be improved by introducing genes or groups of genes from other crop species, algae, and photosynthetic bacteria (Gimpel et al., 2016; Hanson et al., 2016; Sharwood et al., 2016).

Expression of plastid transgenes throughout the plant is desirable for some applications, for example tolerance to herbicides such as phosphinothricin (PPT) (Lutz et al., 2001; Ye et al., 2003), glyphosate (Ye et al., 2003), sulfonylurea, pyrimidinylcarboxylate (Shimizu et al., 2008) and diketonitrile (Dufourmantel et al., 2007). Equally useful are plastid expression of insecticidal protein genes (US 5,545,818) and double-stranded RNAs that are toxic to insects (Zhang et al., 2015). The herbicide resistance and insecticidal genes are introduced by linkage to the selective spectinomycin resistance (*aadA* gene) marker. When uniform transformation of plastid genomes is obtained, the marker gene can be excised by a site-specific recombinase that targets sites flanking the marker gene. Various marker excision systems are suitable including the Cre/loxP or PhiC31/Int systems (as described in US Patents 7,217,860 and 8,841,511) or the BxB1 (Shao et al., 2014), ParA-MRS, and CinH-Rs2 (Shao et al., 2017) site-specific recombination systems.

Particularly effective for the recovery of transplastomic events are the PrnLatpB/TrbcL, PrnLatpB/TpsbA, PrnLrbcL/TpsbA, PrnLT7g10/TrbcL promoter/terminator cassettes (Kuroda and Maliga, 2001, 2001). Genes of interest may also be expressed using cassettes previously described in US Patents 5,977,402, 6,297,054, 6,376,744, 6,472,568, 6,624,296, 6,987,215, 7,176,355, 8,143,474. Figure 10 shows a schematic design of a plastid transformation vector having a *Brassica napus* plastid targeting sequence containing the *rrn16* targeting region (nucleotides 135473-137978 in GenBank accession KP161617) and carrying a recombinase target site-flanked selectable *aadA* marker and a gene of interest.

Tissue-specific expression of plastid genes is desirable but thus far no practical system has been available to achieve this objective. We describe here seed-specific expression of proteins in plastids based on a transgene incorporated in the plastid genome that is regulated by a nuclear gene with a seed-specific promoter. The elements of the system are depicted in Figure 11A. In a *Brassica* spp. the transgene encoding green fluorescent protein (or particular gene of interest) is present in the leaf cell, but is not translated in the absence of a modified PPR10 RNA binding protein. The engineered *Zea mays* PPR10GG protein gene that is required for expression is present in the nucleus, but is not active because it is under the control of a seed-specific *Brassica napus* napin gene promoter that is not transcribed in the nucleus of leaf cells (Ellerstrom et al., 1996). The native *Brassica* PPR10 protein (Bn-PPR10) stabilizes and facilitates translation of the *atpH* mRNA. However, Bn-PPR10 RNA binding protein does not recognize PBS^{ZmGG}, the mutant maize PPR10 binding site because the 23-nucleotide *Brassica*

binding site differs by 2 nucleotides from the wild-type maize PPR10 binding site and by 4 nucleotides from the mutant maize binding site.

Zm-PPR10 wt Binding site: ATTGTATCcTTAACcATTTCTTT (SEQ ID NO: 259)

Bn-PPR10 wt Binding site: ATTGTATCATTAACcATTTCTTT (SEQ ID NO: 260)

5 Zm-PPR10^{GG} mut Binding site: ATTGTAggcTTAACcATTTCTTT (SEQ ID NO: 261)

In the *Brassica* ssp. seed (embryo) cell, the napin seed storage protein gene promoter is turned on, the mRNA is translated in the cytoplasm and the PPR10^{GG} protein is imported into chloroplasts where it binds to its cognate binding site upstream of the *gfp* AUG translation initiation codon. Binding of the Zm-PPR10^{GG} stabilizes the *gfp* mRNA and facilitates its translation. The result is high-level GFP protein accumulation in the plastids of embryo cells in oilseed crops.

To construct the regulated plastid transgenes, the tobacco Prn promoter is linked up with the 100 nt sequence directly upstream of the maize *atpH* gene. The two sequences together constitute the 5' regulatory region driving GFP expression. The *gfp* coding region is followed by the *rbcL* gene terminator (TrbcL). Prn-PPR10^{GG}- GFP-TrbcL corresponds to SEQ ID NO. 262. The transgene is cloned adjacent to an *aadA* gene in the *B. napus*-specific plastid transformation vector shown in Figure 12A. Also shown in Figure 12A is a variant, where a T-RNA (symbolized with a cloverleaf; SEQ ID NO. 263) is cloned between the promoter and the 100 nt maize sequence. The tRNA is efficiently processed to create a processed end that is more sensitive to degradation in the absence of the protecting Zm-PPR10^{GG} protein, reducing background in the absence of the PPR10^{GG} protein. This construct can be engineered to express a protein of interest in the place of GFP, or a protein of interest can be operably linked to GFP via cleavable protein linker.

Likewise, a *Brassica napus* seed-specific PnpA:PPR10^{GG}:Tocs nuclear transgene can be cloned into a pCAMBIA2300 *Agrobacterium* binary vector with a plant-selectable kanamycin resistance gene for transformation of the *B. napus* nucleus (Figure 12B). The modified *Zea mays* PPR10 gene sequence that results in selective recognition of the modified GG RNA binding site is described (Barkan et al., 2012) (SEQ ID NO: 265). For reference, the wild-type maize PPR10 sequence is also listed (SEQ ID NO: 264). The PPR10 protein is naturally targeted to chloroplasts, thus it requires only a tissue-specific promoter and a eukaryotic transcription terminator, such as octopine synthase 3' UTR (Tocs) (GenBank accession no. AJ311872.1). The

napin gene is encoded in a small gene family. A suitable promoter for the PnpA:PPR10^{GG}:Tocs gene was characterized experimentally (Ellerstrom et al., 1996) (GenBank accession J02798), and additional *B. napus* promoters are available (Sohrabi et al., 2015). The promoter of a legume storage protein gene, phaseolin, (SEQ ID NO: 266) is known to be very efficient for the expression of recombinant proteins in *Arabidopsis thaliana* (De Jaegert et al., 2002). The promoter sequence is available in US patent application 2003/0159183.

>Prn-PPR10GG-GFP-TrbcL SEQ ID NO: 262

>GagctcGCTCCCCCGCCGTCGTTCAATGAGAATGGATAAGAGGCTCGTGGGATTGAC

10 GTGAGGGGGCAGGGATGGCTATATTTCTGGGAGTTACTTCTACCCGATAGAGCTTAG
AAGTTGGAAGTAATAATTTCTTGGTTGATTGTAGGCTTAACCATTCTTTTTTTTTGA
CACGAGGAACATCATGgctagcAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCC
AATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGA
GGGTGAAGGTGATGCAACATACGGAAAACCTTACCCTTAAATTTATTTGCACTACTGG
15 AAAACTACCTGTTCCtTGGCCAACACTTGTCCTACTTTCTCTTATGGTGTTCAATGCT
TTTCAAGATACCCAGATCATATGAAGCGGCACGACTTCTTCAAGAGCGCCATGCCTG
AGGGATACGTGCAGGAGAGGACCATCTCTTTCAAGGACGACGGGAACCTACAAGACA
CGTGCTGAAGTCAAGTTTGAGGGAGACACCCTCGTCAACAGGATCGAGCTTAAGGG
AATCGATTTCAAGGAGGACGGAAACATCCTCGGCCACAAGTTGGAATACAACCTACA
20 ACTCCCACAACGTATACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAAC
TTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCA
ACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTC
CACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCT
TGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTgTACAAATAAAAtc
25 tagaAAACAGTAGACATTAGCAGATAAATTAGCAGGAAATAAAGAAGGATAAGGAGA
AAGAACTCAAGTAATTATCCTTCGTTCTCTTAATTGAATTGCAATTAACTCGGCCC
AATCTTTTACTAAAAGGATTGAGCCGAATACAACAAAGATTCTATTGCATATATTTT
GACTAAGTATATACTTACCTAGATATACAAGATTTGAAATACAAAATCTAaagctt

30 **>tRNA (trnP, 147bp) SEQ ID NO: 263**

AAGTCTTTACAATGACAATGGAAACCGATGTAAAGGGATGTAGCGCAGCTTGGTAG
CGCGTTTGT TTTGGGTACAAAATGTCACAGGTTCAAATCCTGTCATCCCTATCCCTAA
CTtGTAGTTATCGTATCAGCAGTAACAATAGAT (SEQ ID NO: 263)

5 >**Zm_PPR10 WT** SEQ ID NO: 264

ATGGAGGCCACCGGCAGGGGGCTGTTCCCGAACAAGCCCACCCTCCCGGCGGGGCC
GAGGAAACGGGGCCCGCTCCTCCCGGCCGCGCCCCCGCCACCGTCCCCCTCCTCGCT
CCCGCTCGACTCGCTCCTGCTCCACCTCACCGCGCCCCGCCCCGCGCCGGCCCCCGC
GCCGCGGCGGTGCGACCAGACGCCGACGCCGCGCACTCCTTCCTCTCCCCCGACGC
10 GCAGGTGCTGGTGCTCGCCATCTCCTCGCACCCGCTCCCCACGCTGGCGGCCTTCCT
GGCCTCCCGCCGCGACGAGCTCCTCCGCGCGGACATCACGTCCCTGCTCAAGGCGCT
GGAGCTCTCGGGGCACTGGGAGTGGGCGCTCGCGCTCCTCCGGTGGGCAGGCAAGG
AGGGTGCCGCCGACGCGTCGGCGCTCGAGATGGTCGTCCGCGCGCTGGGCCGCGAG
GGCCAGCACGACGCCGTCTGCGCGCTGCTCGACGAAACGCCGCTCCCGCCGGGCTC
15 CCGCCTCGACGTCCGCGCCTACACCACCGTGCTGCACGCGCTCTCCCGCGCGGGCCG
GTACGAGCGCGCGCTCGAGCTCTTCGCCGAGCTCCGGCGCCAGGGGGTGGCGCCCA
CGCTCGTCACCTACAACGTCGTGCTGGACGTGTACGGGCGGATGGGCCGGTTCGTGG
CCGCGGATCGTCGCCCTCCTCGATGAGATGCGCGCCGCCGGGGTCGAGCCCGACGG
CTTCACCGCCAGCACGGTGATCGCCGCGTGCTGCCGCGACGGGCTGGTTGACGAGG
20 CGGTGGCGTTCTTCGAGGACCTCAAGGCCCGCGGCCACGCCCCGTGCGTCGTACGTC
ACaacGCGTTGCTCCAGGTGTTTCGGCAAGGCCGGGAACCTACACGGAGGCGCTGCGCG
TGCTCGGGGAGATGGAGCAGAACGGCTGCCAGCCAGATGCTGTGACGTACaacGAGC
TCGCCGGAACGTACGCCCGGGCTGGGTTCTTCGAGGAGGCTGCCAGGTGCCTGGAC
ACAATGGCATCCAAGGGTCTGTTGCCAAACGCATTACGTACAACACCGTGATGAC
25 AGCCTATGGGAATGTTGGGAAGGTGGATGAGGCGCTCGCTCTGTTTGACCAGATGA
AGAAGACCGGGTTCGTGCCGAACGTGAACACGTACAATCTTGTCCTTGGCATGCTTG
GCAAGAAGTCAAGGTTACGGTGATGCTAGAGATGCTTGAGAGATGTCGAGGAGC
GGATGCACACCGAACCGGGTAACATGGAACACAATGCTTGCAGTCTGTGGGAAGCG
TGGCATGGAGGACTACGTCACCCGGGTTCTGGAGGGGATGAGGTCTTGCGGGGGTTG
30 AACTGAGCCGAGACACCTACAACACCCTGATAGCTGCGTACGGCCGGTGTGGCTCG
AGGACTAATGCCTTCAAGATGTACAACGAGATGACCAGCGCTGGATTACCCCCCTG

CATCACCACGTACAACGCGTTGCTGAACGTGCTGTCGCGGCAGGGCGACTGGTCCA
CCGCCCAGTCGATCGTAAGCAAAATGAGGACCAAGGGGTTCAAGCCGAACGAGCAG
TCGTATTCGCTGCTGCTCCAGTGCTACGCGAAGGGGGGCAACGTGGCAGGGATAGC
CGCGATCGAGAACGAGGTGTACGGATCAGGTGCCGTTTTCCCAAGCTGGGTGATCCT
5 GAGGACCCTTGTCATCGCCAATTTCAAGTGCCGGCGACTGGATGGCATGGAGACGG
CGTTTCAAGAGGTGAAGGCCAGAGGCTACAACCCGGACCTCGTGATATTCAACTCC
ATGCTGTCCATCTACGCGAAGAACGGGATGTACAGCAAGGCCACCGAGGTCTTCGA
CTCCATCAAGCGGAGCGGGCTGAGCCCCGACCTCATCACCTACAACAGCCTGATGG
ACATGTACGCCAAGTGCAGCGAGTCGTGGGAGGCCGAGAAGATACTGAACCAGCTC
10 AAGTGCTCCCAGACGATGAAGCCCGACGTGGTGTCTACAACACGGTCATAAACGG
GTTCTGCAAGCAGGGGCTGGTGAAAGAGGCCAGAGGGTCCTCTCGGAGATGGTCG
CCGACGGCATGGCCCCCTGCGCCGTGACCTACCACACGCTCGTCGGGGGTACTCCA
GCCTGGAGATGTTACGCGAGGCCAGGGAGGTCATCGGCTACATGGTCCAGCACGGC
CTCAAGCCTATGGAGCTGACCTACAGGAGAGTCGTCGAGAGCTACTGCAGAGCGAA
15 GCGGTTTCGAGGAGGCTCGCGGCTTCCTGTCCGAGGTCTCGGAGACCGACCTGGATTT
TGACAAGAAGGCGCTCGAAGCCTATATAGAGGATGCGCAGTTTGGAAGGTAG (SEQ

>Zm_PPR10 GG SEQ ID NO: 265

ATGGAGGCCACCGGCAGGGGGCTGTTCCCGAACAAGCCCACCTCCCGGCGGGGCC
20 GAGGAAACGGGGCCCGCTCCTCCCGGCCGCGCCCCCGCCACCGTCCCCCTCCTCGCT
CCCGCTCGACTCGCTCCTGCTCCACCTCACCGCGCCCGCCCCGCGCCGGCCCCCGC
GCCGCGGCGGTGCGACCAGACGCCGACGCCGCCGCACTCCTTCCTCTCCCCGACGC
GCAGGTGCTGGTGCTCGCCATCTCCTCGCACCCGCTCCCCACGCTGGCGGCCTTCCT
GGCCTCCCGCCGCGACGAGCTCCTCCGCGCGGACATCACGTCCCTGCTCAAGGCGCT
25 GGAGCTCTCGGGGCACTGGGAGTGGGCGCTCGCGCTCCTCCGGTGGGCAGGCAAGG
AGGGTGCCGCCGACGCGTCGGCGCTCGAGATGGTCGTCCGCGCGCTGGGCCGCGAG
GGCCAGCACGACGCCGTCTGCGCGCTGCTCGACGAAACGCCGCTCCCGCCGGGCTC
CCGCCTCGACGTCCGCGCCTACACCACCGTGCTGCACGCGCTCTCCCGCGCGGGCCG
GTACGAGCGCGCGCTCGAGCTCTTCGCCGAGCTCCGGCGCCAGGGGGTGGCGCCCA
30 CGCTCGTCACCTACAACGTCGTGCTGGACGTGTACGGGCGGATGGGCCGGTCTGTGG
CCGCGGATCGTCGCCCTCCTCGATGAGATGCGCGCCGCCGGGGTTCGAGCCCGACGG

CTTACCCGCCAGCACGGTGATCGCCGCGTGCTGCCGCGACGGGCTGGTTGACGAGG
CGGTGGCGTTCTTCGAGGACCTCAAGGCCCGCGGCCACGCCCCGTGCGTCGTACGT
ACacaGCGTTGCTCCAGGTGTTCTGGCAAGGCCGGGAACCTACACGGAGGCGCTGCGCG
TGCTCGGGGAGATGGAGCAGAACGGCTGCCAGCCAGATGCTGTGACGTACaccGAGC
5 TCGCCGGAACGTACGCCCCGGGCTGGGTTCTTCGAGGAGGCTGCCAGGTGCCTGGAC
ACAATGGCATCCAAGGGTCTGTTGCCAAACGCATTCACGTACAACACCGTGATGAC
AGCCTATGGGAATGTTGGGAAGGTGGATGAGGCGCTCGCTCTGTTTGACCAGATGA
AGAAGACCGGGTTCGTGCCGAACGTGAACACGTACAATCTTGTCCTTGGCATGCTTG
GCAAGAAGTCAAGGTTACGGTGATGCTAGAGATGCTTGGAGAGATGTCGAGGAGC
10 GGATGCACACCGAACC GGTAACATGGAACACAATGCTTGCAGTCTGTGGGAAGCG
TGGCATGGAGGACTACGTCACCCGGGTTCTGGAGGGGATGAGGTCTTGCGGGGTTG
AACTGAGCCGAGACACCTACAACACCCTGATAGCTGCGTACGGCCGGTGTGGCTCG
AGGACTAATGCCTTCAAGATGTACAACGAGATGACCAGCGCTGGATTCACCCCCTG
CATCACACGTACAACGCGTTGCTGAACGTGCTGTCGCGGCAGGGCGACTGGTCCA
15 CCGCCCAGTCGATCGTAAGCAAAATGAGGACCAAGGGGTTCAAGCCGAACGAGCAG
TCGTATTCGCTGCTGCTCCAGTGCTACGCGAAGGGGGGCAACGTGGCAGGGATAGC
CGCGATCGAGAACGAGGTGTACGGATCAGGTGCCGTTTTCCCAAGCTGGGTGATCCT
GAGGACCCTTGTCATCGCCAATTTCAAGTGCCGGCGACTGGATGGCATGGAGACGG
CGTTTCAAGAGGTGAAGGCCAGAGGCTACAACCCGGACCTCGTGATATTCAACTCC
20 ATGCTGTCCATCTACGCGAAGAACGGGATGTACAGCAAGGCCACCGAGGTCTTCGA
CTCCATCAAGCGGAGCGGGCTGAGCCCCGACCTCATCACCTACAACAGCCTGATGG
ACATGTACGCCAAGTGCAGCGAGTCGTGGGAGGCCGAGAAGATACTGAACCAGCTC
AAGTGCTCCCAGACGATGAAGCCCGACGTGGTGTCTTACAACACGGTCATAAACGG
GTTCTGCAAGCAGGGGCTGGTGAAAGAGGGCCAGAGGGTCCTCTCGGAGATGGTCG
25 CCGACGGCATGGCCCCCTGCGCCGTGACCTACCACACGCTCGTCGGGGGTTACTCCA
GCCTGGAGATGTTTACGCGAGGCCAGGGAGGTCATCGGCTACATGGTCCAGCACGGC
CTCAAGCCTATGGAGCTGACCTACAGGAGAGTCGTGAGAGCTACTGCAGAGCGAA
GCGGTTTCGAGGAGGCTCGCGGCTTCCTGTCCGAGGTCTCGGAGACCGACCTGGATTT
TGACAAGAAGGCGCTCGAAGCCTATATAGAGGATGCGCAGTTTGGAAGGTAG (SEQ

30

Phaseolin promoter SEQ ID NO: 266

ggctgacgggtatcgataagcttgatatcgaattcctgcagcccaattcattgtactcccagtatcattatagtgaaagtttggctctcgcggg
 tgggtttttacctctatttaaaggggtttccacctaataattctggtatcattctcactttactgttactttaatttcataatcttgggtgaaattatc
 acgcttccgcacacgatatccctacaaattatttattgttaaacaatttcaaaccgcataaaatttatgaagtcctctatctttaatgtagtctaa
 cattttcatattgaaatatataatttacttaattttagcgttggttagaaagcataatgatttattcttattcttctcatataaattgtaataatacaat
 5 ataaacaaattctttacctaagaaggatttccattttatattttaaaaatataatttatcaaatattttcaaccacgtaaatctcataataaagttgt
 ttcaaaagtaataaaatttaactccataattttttattcgactgatcttaaagcaacaccagtgacacaactagccattttttcttggataaaaa
 aatccaattatcattgtattttttatatacaatgaaaatttcaccaaacacattgtgtgatttctgaagcaagtcattgtatgcaaaattctataatt
 cccatttgacactacggaagtaactgaagatctgcttttacctgcgagacacatcttctaaagtaattttaataatagttactatattcaagatttca
 tatatcaaaactcaatattacttctaaaaaatttaattagatataaataaataattacttttttaattttaagttaattgttgaattgtgactattgatttatt
 10 attctactatgtttaaattgtttatagatagtttaaagtaaatataagtaattgtagtagagtgttagagtgttacctaaccataaaactataaacattt
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 gctcacccatctcaaccacacacaaacacattgcctttttcttcatcatcaccacaaccacctgtatatattcttcttccgccacctcaatttc
 15 ttcaattcaacacacgtcaacctgca

The *Arabidopsis* nuclear genome encodes >400 Pentatricopeptide Repeat Proteins (PPRs), of which PPR10 is a member (Barkan and Small, 2014). Other P-type proteins that function similar to PPR10 are the *Arabidopsis* HCF152 and PGR3 proteins which is required for the
 20 accumulation of transcripts cleaved in the *psbH-petB* intergenic region and *petL* operon, respectively (Meierhoff et al., 2003; Yamazaki et al., 2004). *Zea mays* CRP1 is involved in the processing and translation of the chloroplast *petD* and *petA* RNAs (Fisk et al., 1999). HCF107, a member in the half-a-tetratricopeptide (HAT) family, also defines the processed end of *psbH* and enhance its translation by remodeling its 5' UTR (Hammani et al., 2012). These proteins with
 25 their cognate binding site can be engineered to test and establish similar chloroplast transgene regulation system as PPR10.

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While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

What is claimed is:

1. A method for increasing sensitivity to spectinomycin in plastids of higher plants for increasing plastid transformation efficiency, comprising;

a) providing a plant comprising a nonfunctional ACC2 nuclear gene;

b) introducing one or more plastid transformation vectors into the plastids in cells from said plant, said one or more vectors comprising an aadA spectinomycin resistance marker sequence and a nucleic acid sequence encoding a protein of interest;

c) contacting said cells with spectinomycin and selecting plant cells which are resistant to spectinomycin and accumulate said protein of interest in said plastids; and

d) culturing said plant cells under conditions suitable to regenerate a transplastomic plant therefrom.

2. The method of claim 1, wherein said plant is selected from the group consisting of *Arabidopsis* ssp., *Brassica* ssp., and *Camelina* ssp.

3. The method of claim 1, wherein said protein of interest is green fluorescent protein.

4. The method of claim 1, wherein the plant of step a) is a naturally occurring mutant which encodes non-functional or defective ACC2.

5. The method of claim 1, wherein said ACC2 gene is inactivated in said plant using CRISPR/Cas prior to plastid transformation.

6. The method of claim 5, wherein said plant is a *Brassica* ssp. plant.

7. The method of claim 5, wherein said plant is a *Camelina* ssp. plant.

8. The method of claim 1, further comprising excising said aadA spectinomycin resistance marker sequence from said plant.

9. The method of claim 5, wherein said protein of interest is selected from the group consisting of a protein conferring herbicide resistance, a protein conferring insect resistance, a vaccine, an antibody, regulatory RNA, dsRNA, siRNA, shRNA and insecticidal proteins.

5 10. A method for seed-specific plastid expression comprising:

a) introducing a nuclear expression vector encoding a modified PPR10 binding protein driven by a seed-specific promoter and

b) a plastid expression vector encoding a gene of interest linked to an upstream PPR10 binding site, wherein nuclear-expressed PPR10 is imported into plastids and binds said PPR10
10 binding site to drive expression of the gene of interest in seed plastids.

11. The method of claim 10, wherein said vector comprises a seed specific promoter selected from a napin or a phaseolin gene promoter.

12. The method of claim 10, wherein said modified PPR10 binding protein is PPR10^{GG} encoded by SEQ ID NO: 265.

15 13. The method of claim 10, wherein said PPR10 binding site encoded by SEQ ID NO: 261.

14. The method of claim 10, further comprising plastid expression of an *aadA* spectinomycin resistance gene.

15. The method of claim 10, wherein the plastid expressed gene of interest is linked to an upstream sequence encoding a maize *atpH* gene and/or tRNA sequence in said plastid vector.

20 16. A method for increasing sensitivity to plastid translation inhibitors in plastids of higher plants for increasing plastid transformation efficiency, comprising;

a) providing a plant comprising a nonfunctional ACC2 nuclear gene;

b) introducing one or more plastid transformation vectors into the plastids in cells from said plant, said one or more vectors comprising a nucleic acid sequence conferring resistance to
25 said plastid translation inhibitor, and a nucleic acid sequence encoding a protein of interest;

c) contacting said cells with said inhibitor and selecting plant cells which are resistant to said inhibitor and accumulate said protein of interest in said plastids; and

d) culturing said plant cells under conditions suitable to regenerate a transplastomic plant therefrom.

17. The method of claim 16, wherein said plastid translation inhibitor is selected from the group
5 consisting of kanamycin, chloramphenicol, tobramycin and gentamycin.

18. The method of claim 17, wherein inhibitor is kanamycin.

19. The method of claim 17, wherein said inhibitor is chloramphenicol and said nucleic acid
10 encodes chloramphenicol acetyl transferase.

20. The method of claim 17, wherein said inhibitor is tobramycin.

21. The method of claim 17, wherein said inhibitor is gentamycin.

15

The diagram illustrates the chloroplast ACCase pathway and its regulation. It is divided into three main regions: Chloroplast, Cytoplasm, and Nucleus.

- Chloroplast:** Contains the *accD* gene, which is transcribed into mRNA and translated into hetACCase. hetACCase is a complex of proteins including 8-CT, 8-CT, ACCP, and 8C. The chloroplast is separated from the cytoplasm by a double membrane, with TIC/TOC complexes embedded in it.
- Cytoplasm:** Contains the *acc1* gene, which is transcribed into mRNA and translated into homoACCase. The cytoplasm is separated from the nucleus by a nuclear envelope.
- Nucleus:** Contains several TP-CAC genes (TP-CAC1-A, TP-CAC2, TP-CAC3) and a TP-CAC2 gene that is crossed out. Arrows indicate the flow of information and regulation between these components.

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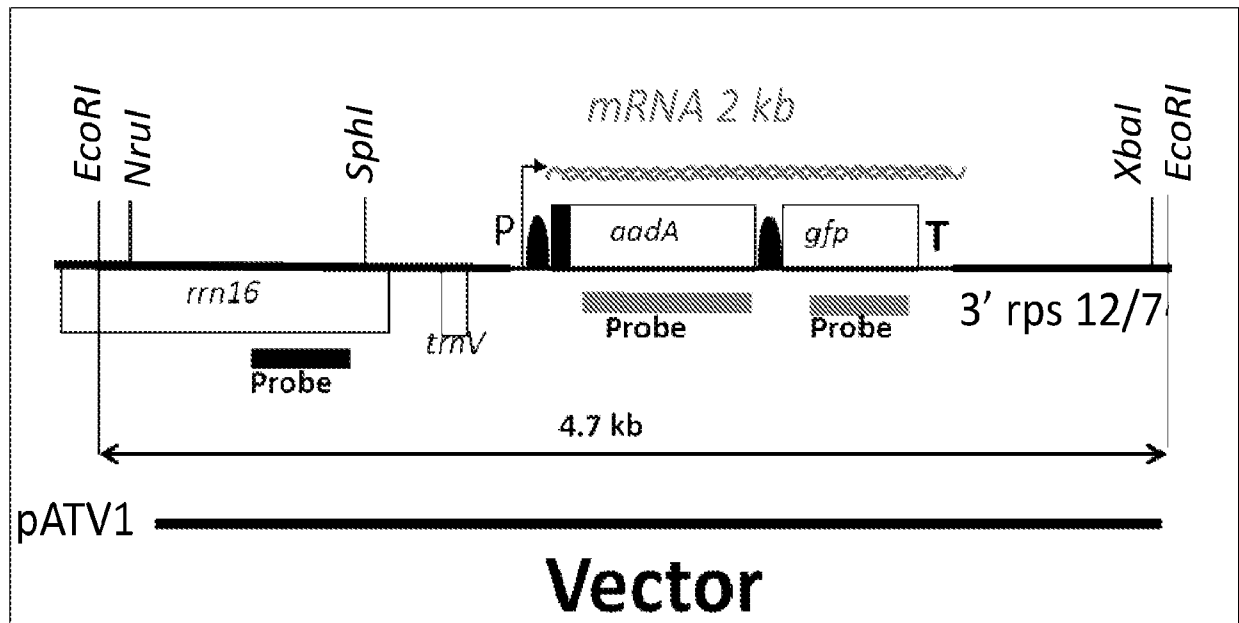


Figure 2

Fig. 3A

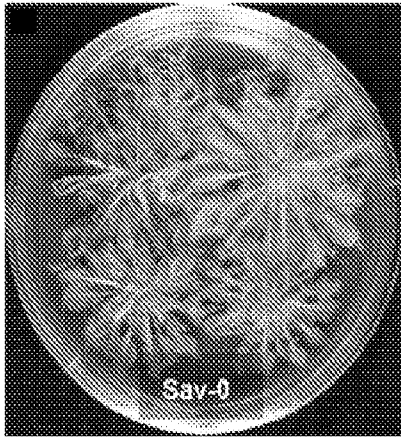


Fig. 3C

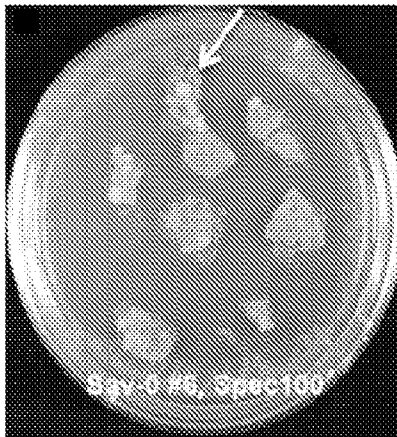


Fig. 3E

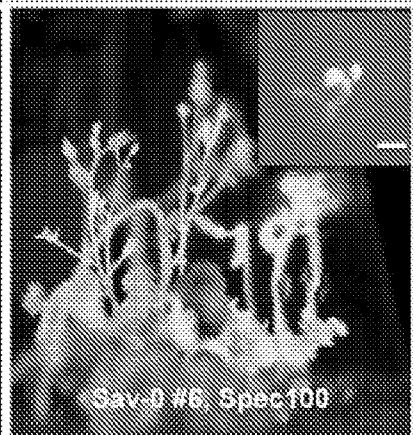
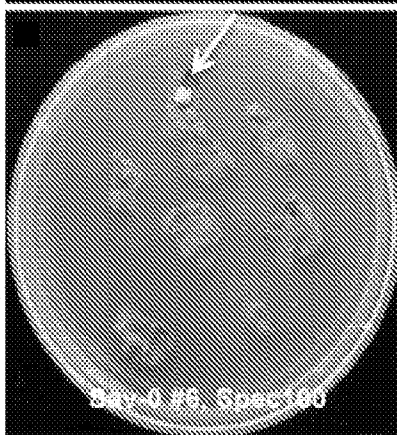
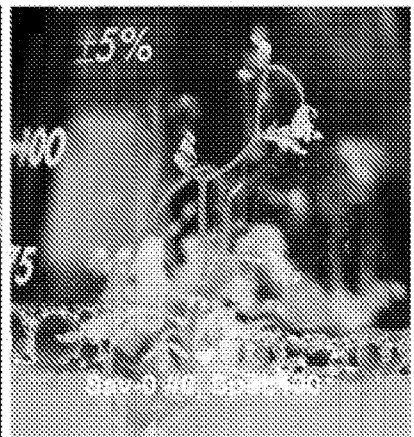


Fig. 3B

Fig. 3D

Fig. 3F

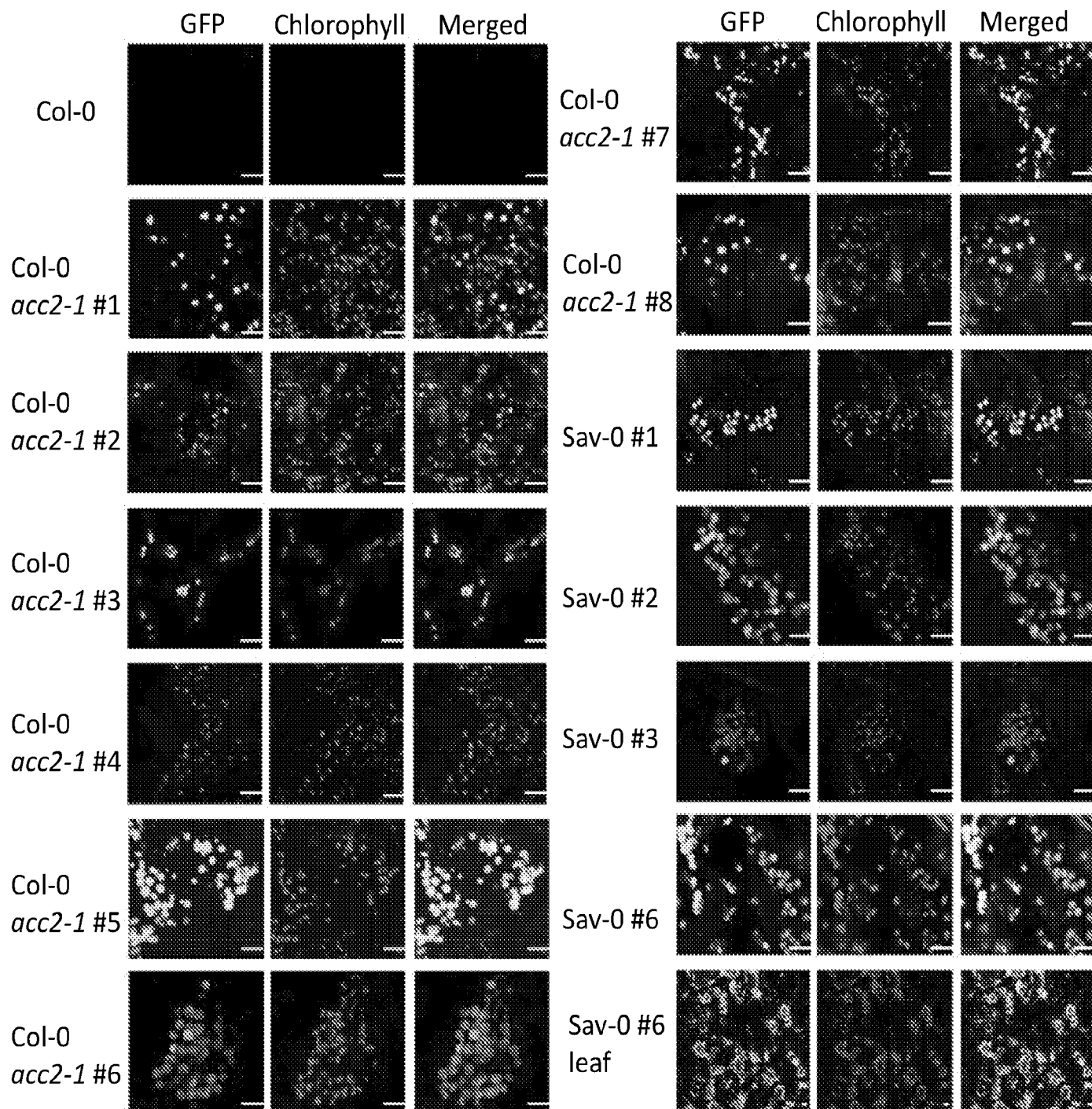


Figure 4

5/12

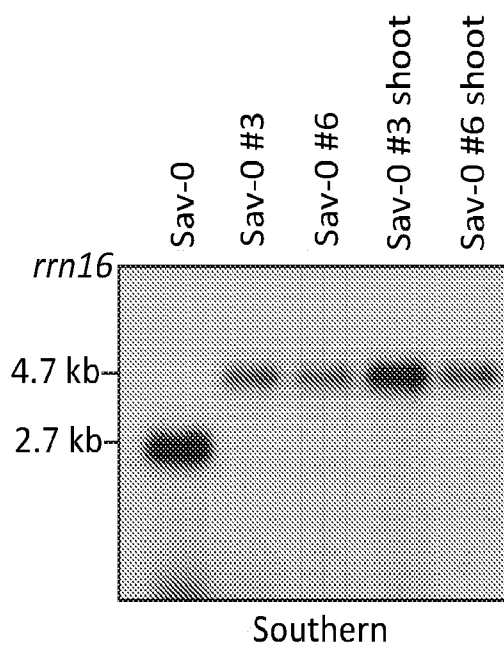
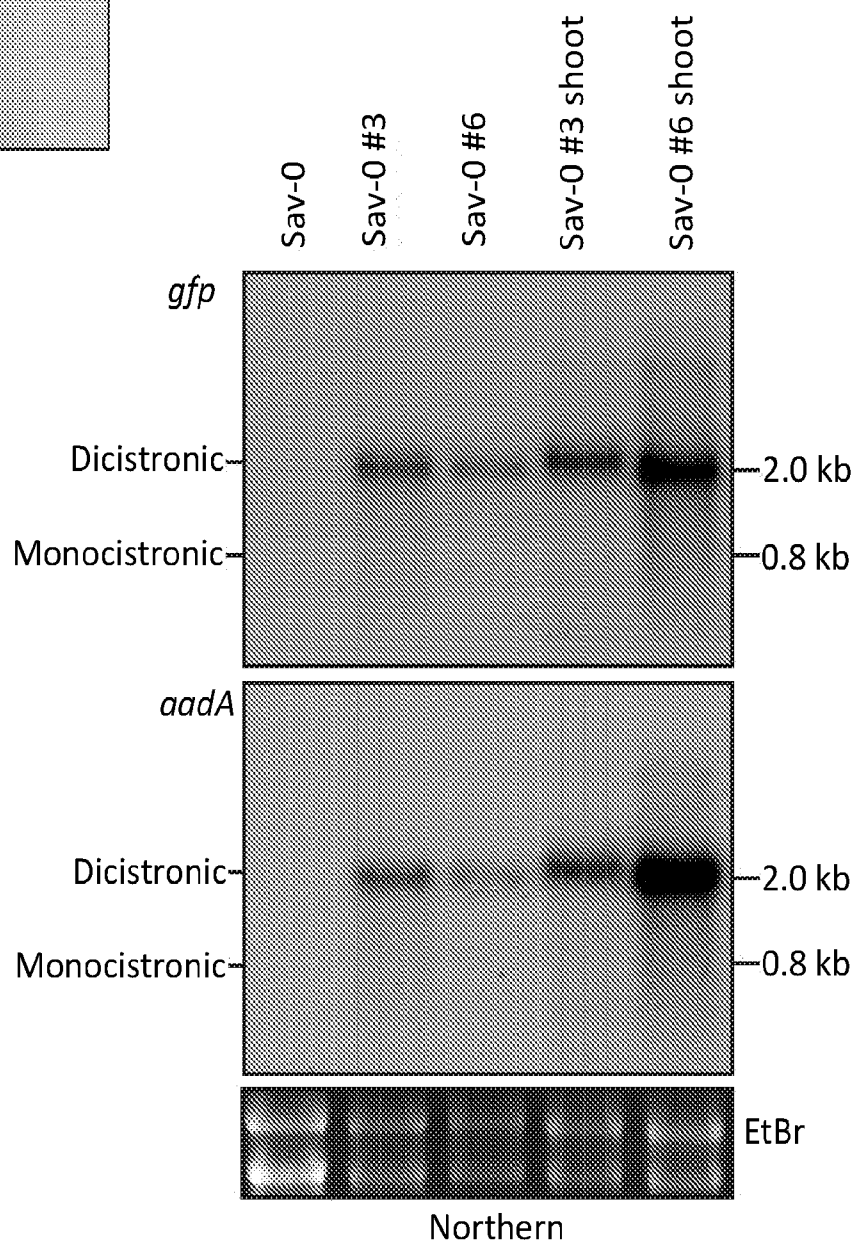


Fig. 5A



Northern

Fig. 5B

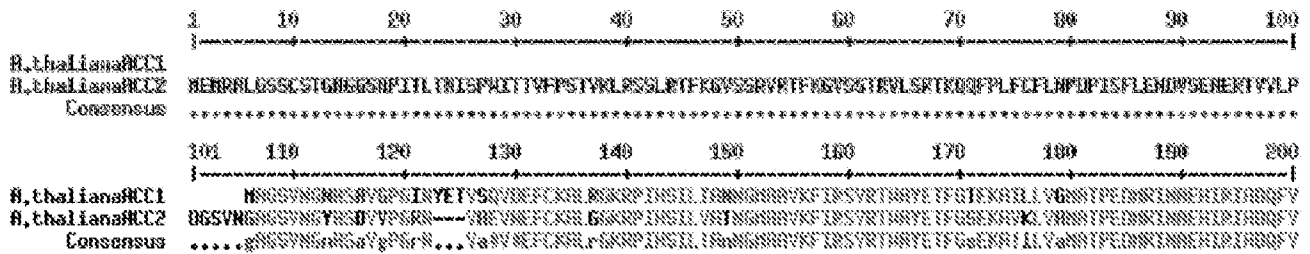


Fig. 6B

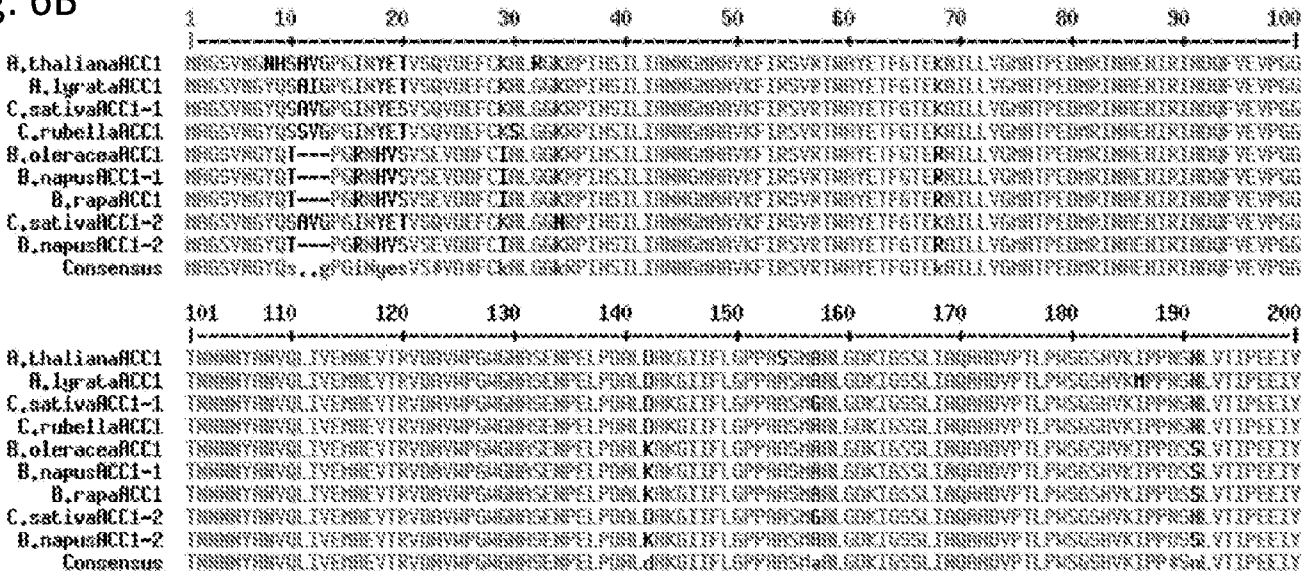
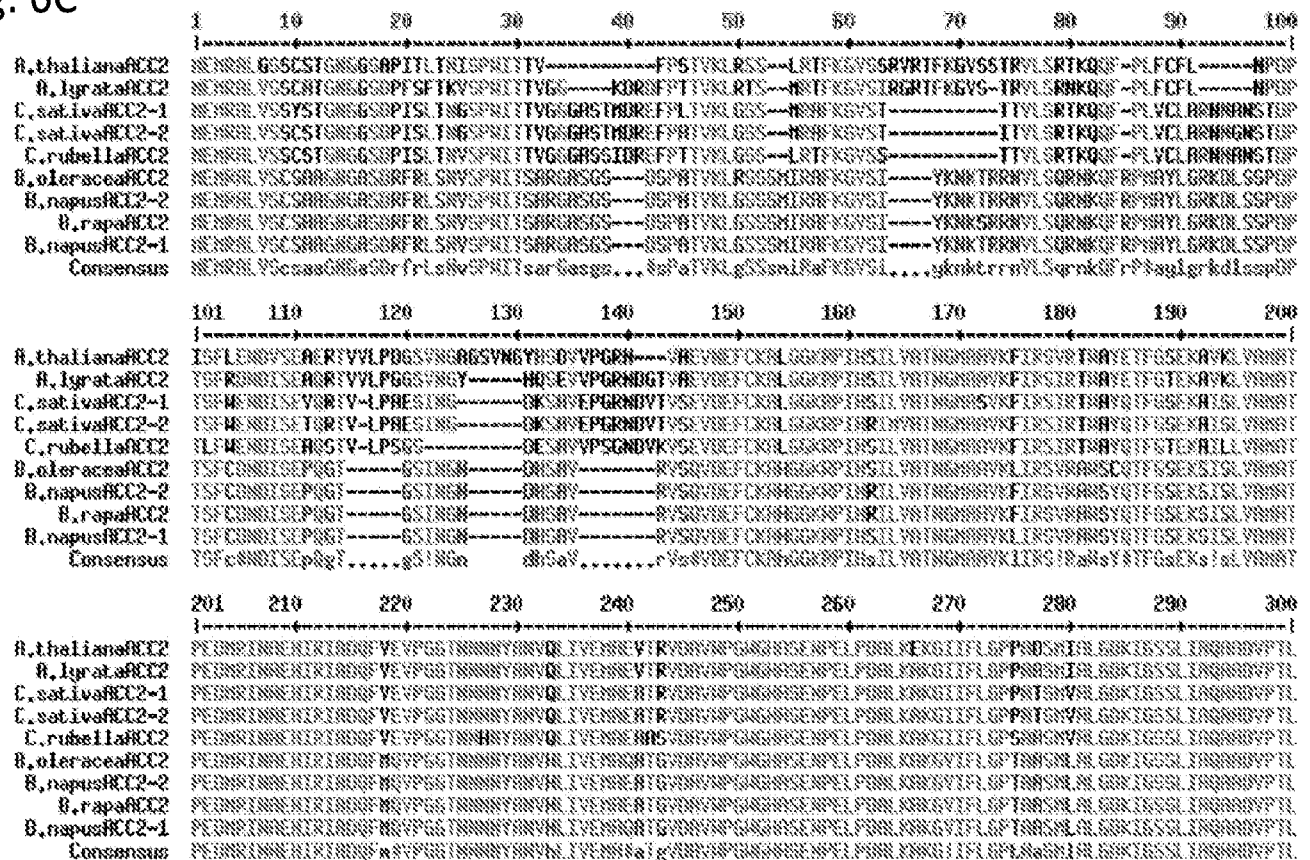


Fig. 6C



7/12

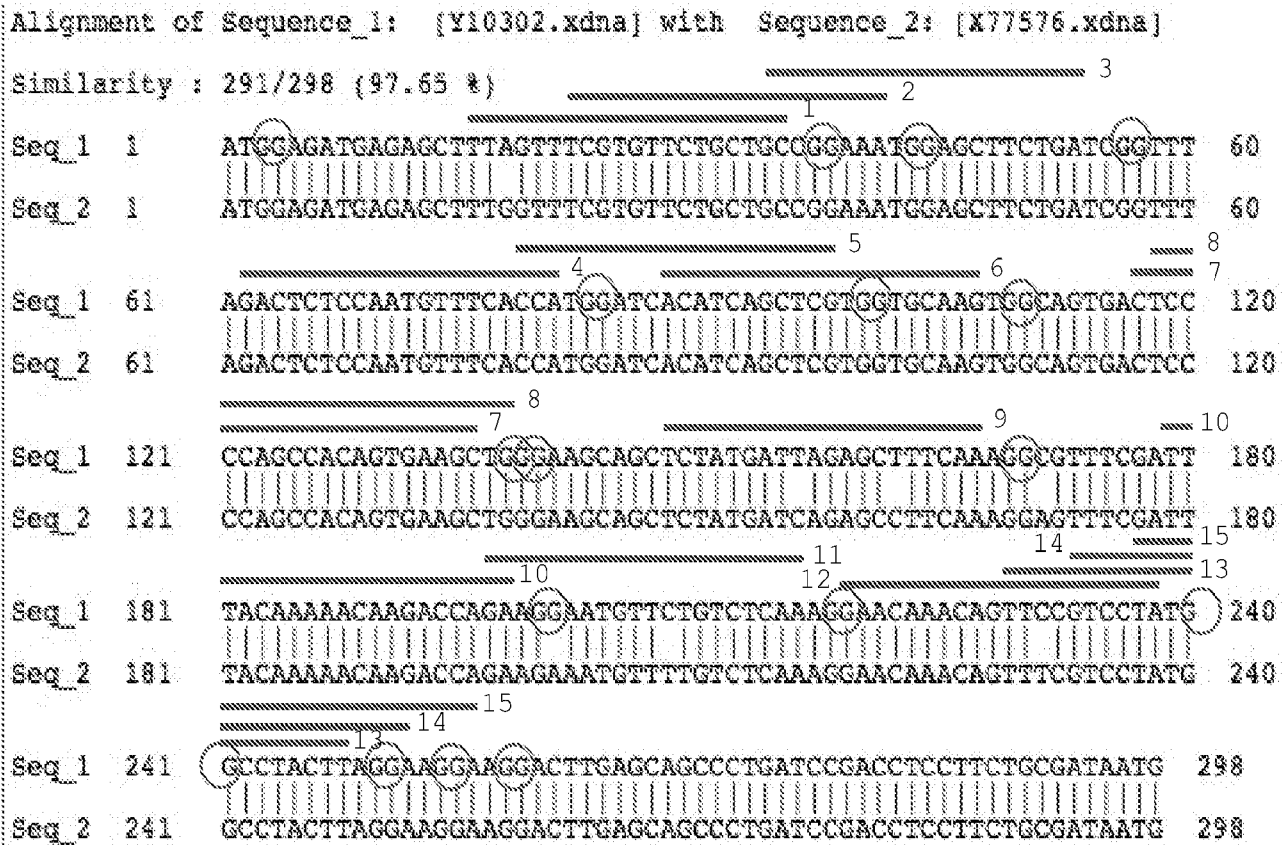


Figure 7

	sgRNA	PAM
Col-0	ATGGA	GGTATTAATGA
Ws/RLD	ATGGA	GGTATTAATGA
Ws-6-2	ATGGA	GGTATTAATGA
Ws-10-35	ATGGA	GGTATTAATGA
Ws-11-5	ATGGA	GGTATTAATGA
Ws-11-28	ATGGA	GGTATTAATGA
Ws-6-23	ATGGA	GGTATTAATGA
Ws-6-11	ATGGA	GGTATTAATGA
Ws-6-9	ATGGA	GGTATTAATGA
Ws-11-46	ATGGA	GGTATTAATGA
Ws-6-19	ATGGA	GGTATTAATGA
Col-0	ATGGA	GGTATTAATGA
Ws/RLD	ATGGA	GGTATTAATGA
RID-6-2	ATGGA	GGTATTAATGA
RID-10-25	ATGGA	GGTATTAATGA
RID-6-10	ATGGA	GGTATTAATGA
RID-10-2	ATGGA	GGTATTAATGA
RID-6-13	ATGGA	GGTATTAATGA
RID-6-15	ATGGA	GGTATTAATGA
RID-10-10	ATGGA	GGTATTAATGA
RID-6-6	ATGGA	GGTATTAATGA
RID-10-29	ATGGA	GGTATTAATGA
RID-11-14	ATGGA	GGTATTAATGA
RID-10-8	ATGGA	GGTATTAATGA

sgRNA-ACC2-F2	Seq 1	1	gattgCCTCAGGAATATATCTCCA	24
sgRNA-ACC2-R2	aaacTGGAGATATATCTGAGGC	1	-----gCCTCAGGAATATATCTCCagttt	24

Figure 8

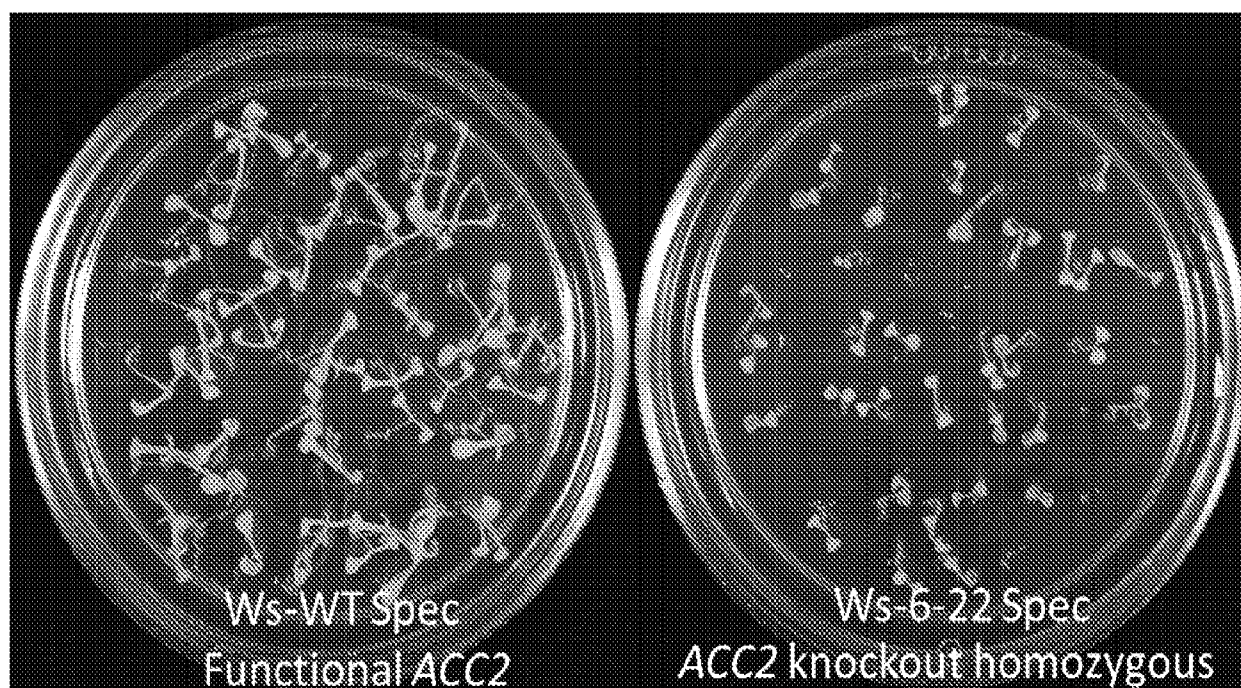


Figure 9

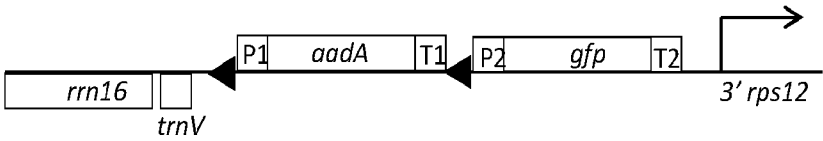


Figure 10

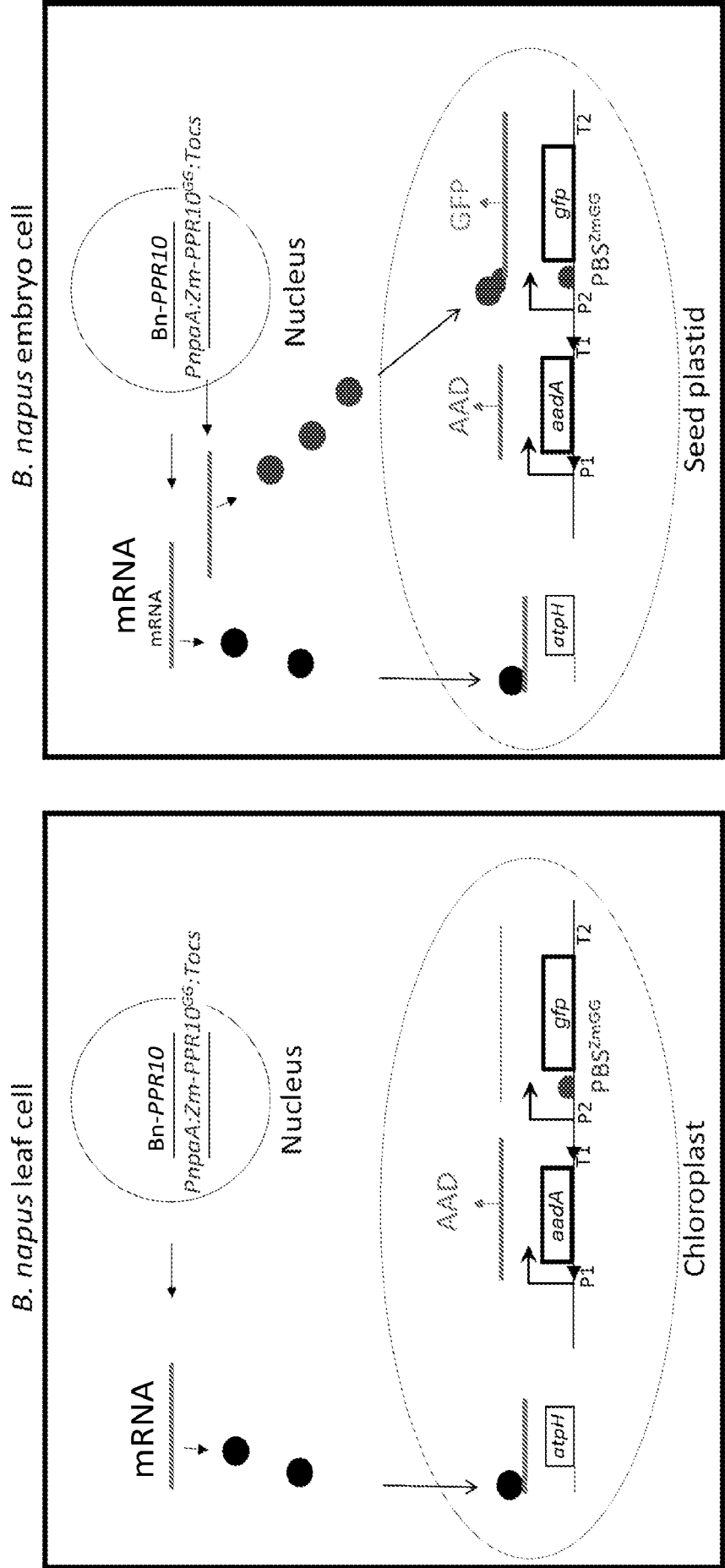


Figure 11

Fig. 12A

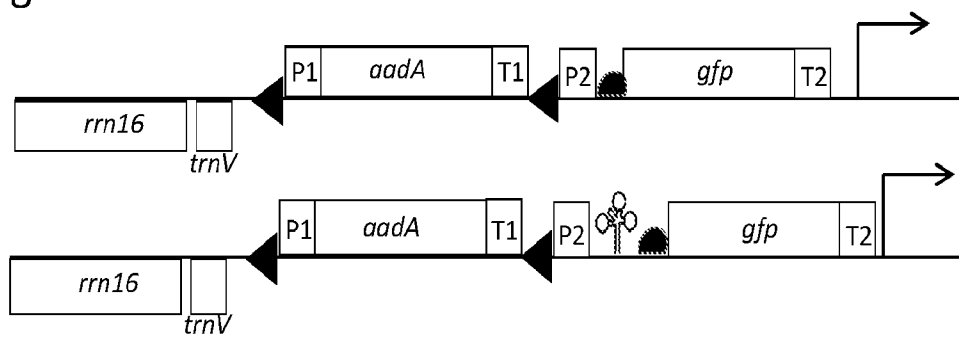
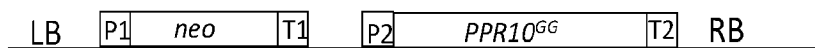


Fig. 12B



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US18/13034

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☐ forming part of the international application as filed:
☐ in the form of an Annex C/ST.25 text file.
☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13*ter*. 1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*. 1(a)).
☐ on paper or in the form of an image file (Rule 13*ter*. 1(b) and Administrative Instructions, Section 713).
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US18/13034

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- Please See Within the Next Supplemental Page-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Group 1 Claims 1-9, 16-21

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US18/13034

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 5/10, 15/09, 15/63, 15/65, 5/82, 9/22, 15/29 (2018.01)

CPC - C12N 5/10, 15/09, 15/63, 15/65, 15/8209, 15/8213, 15/8214, 15/8216, 9/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2003/0200568 A1 (MALIGA, P et al.) 23 October 2003; paragraphs [0007], [0022], [0046]-[0048], [0057], [0060], [0067], [0100]; figure 9; claims 1, 11	1-4, 8-9
Y	(PARKER, N et al.) Natural Variation in Sensitivity to a Loss of Chloroplast Translation in Arabidopsis. Plant Physiology. 21 October, 2014; Vol. 166, No. 4; pages 2013-2027; page 2017; column 2, paragraph 1; DOI: 10.1104/pp.114.249052	1-4, 8-9, 16-21
Y	US 2012/0151627 A1 (MALIGA, P) 14 June 2012; paragraphs [0061], [0111], [0129]-[0130], [0135], [0137]; claims 3, 6, 9	16-21
Y	(LI, W et al.) Chloramphenicol Acetyltransferase as Selectable Marker for Plastid Transformation. Plant Molecular Biology. 19 August, 2010; Vol. 76, No. 3-5; pages 443-451; abstract; page 444, column 1, paragraphs 3-4; DOI: 10.1007/s11103-010-9678-4	19
Y	(TABATABAEI, I et al.) A Bifunctional Aminoglycoside Acetyltransferase/Phosphotransferase Conferring Tobramycin Resistance Provides an Efficient Selectable Marker for Plastid Transformation. Plant Molecular Biology. 17 November, 2016; Vol. 93, No. 3; pages 269-281; abstract; page 273, column 2, paragraph 2; DOI: 10.1007/s11103-016-0560-x	20
P,X	(YU, Q et al.) Efficient Plastid Transformation in Arabidopsis. Plant Physiology. 24 July, 2017; Vol. 175, No. 1; pages 186-193; entire document; DOI: 10.1104/pp.17.00857	1-9, 16-21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

17 May 2018 (17.05.2018)

Date of mailing of the international search report

17 JUN 2018

Name and mailing address of the ISA/

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P.O. Box 1450, Alexandria, Virginia 22313-1450

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Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US18/13034

-***-Continued from Box III Observations where unity of invention is lacking -***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, Claims 1-9 and 16-21 are directed toward methods for increasing sensitivity to spectinomycin and increasing sensitivity to plastid translation inhibitors in plastids of higher plants for increasing plastid transformation efficiency.

Group II, Claims 10-15 are directed toward a method for seed-specific plastid expression.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include a plant comprising a nonfunctional ACC2 nuclear gene, not present in Group II; the special technical features of Group II include a modified PPR10 binding protein, not present in Group I.

Groups I and II share the technical features including: plastids; a vector and expression of a gene of interest in plastids.

However, these shared technical features are previously disclosed by US 2003/0200568 A1 to Maliga et al. (hereinafter 'Maliga').

Maliga discloses plastids (plastids; paragraph [0010]); a vector (a vector; paragraph [0010]) and expression of a gene of interest in plastids (expression of a selectable marker gene and foreign gene of interest in plastids; paragraphs [0005], [0010]).

Since none of the special technical features of the Groups I and II inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Maliga reference, unity of invention is lacking.