Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60580R2
Full Title:	Genetic transformation of plant mitochondria with mTP-DNA nano-complexes
Section/Category:	JoVE Biology
Keywords:	gene delivery, microspores, mitochondria, mTPs, plants, protoplasts, transformation, triticale
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Lethbridge, Alberta, Canada

TITLE:

Genetic Transformation of Plant Mitochondria with mTP-DNA Nano-Complexes

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KEYWORDS

gene delivery, microspores, mitochondria, mTPs, protoplasts, transformation, triticale

SUMMARY

Here, we present a protocol for gene delivery into plant cell mitochondria. This new method utilizes cell penetrating peptides with mitochondria targeting properties and a mitochondria-optimized *aadA:gfp* reporter cassette. It is suitable for transient and stable, organelle-specific gene transfer and expression in somatic and germ cells.

ABSTRACT:

We report a peptide based in vivo plant cell mitochondrial genetic engineering method. Peptide vectors were created by selecting mitochondrial protein sorting signal sequences that contain similar physical and chemical properties as a cell-penetrating peptide (CPP). In the presence of exogenous double-stranded DNA (dsDNA) the peptide vectors called mitochondrial targeting peptides (mTPs) form peptide-nucleic acid nanoparticles. The nanoparticles when incubated with plant cells first cross the cellular membrane and then cross the outer and inner mitochondrial membranes to deliver exogenous DNA into the mitochondria of plant cells. The exogenous DNA integrates into the mitochondrial genome and the transfected plant cells can be cultured into plantlets. A linear double stranded gene construct was delivered into protoplasts and microspores of AC Ultima spring triticale (*X. Triticosecale* Wittmack) plants using the mTP peptide vector system to study in vivo mitochondrial transfection of plant cells and transient mitochondrial gene expression.

INTRODUCTION

Mitochondria are organelles that supply energy for metabolic activities in eukaryotic cells. In plants, the mitochondria are involved not only with energy conversion during aerobic respiration and carbon metabolism in the Krebs cycle, but also to chloroplast function and stress responses[1]. The ability to manipulate the mitochondrial genome will facilitate the study of plant primary and

secondary metabolism. In addition, plant mitochondrial genetic engineering has many important applications in agricultural crop breeding and hybrid maintenance due to the mitochondria being linked to cytoplasmic male sterility[2].

In vivo mitochondrial genetic research in eukaryotic cells has been restricted due to the lack of ability to genetically manipulate the mitochondria in whole cells. Biolistic transformation of *Chlamydomonas reinhardtii* and two yeast species, *Saccharomyces cerevisiae* and *Candida glabrata,* has been successfully applied to mitochondrial transformation[3-5]. A mitochondrial targeted adenoassociated virus has also recently proved to be effective as a mitochondrial DNA delivery method in a mouse model system[6]. In a recent report, a fusion peptide combining a mitochondrial-targeting peptide and cell-penetrating peptide was shown to deliver foreign DNA into the mitochondria of *Arabidopsis thaliana* leaf cells through infiltration[7].

Isolated mitochondria served as the earliest in vitro model for mitochondrial genetic transformation research. An example of this is a study that examined mitochondrial transcription and RNA maturation processes using electroporated wheat isolated mitochondria[8]. Isolated mitochondria have also been transformed utilizing their natural competence to import double stranded linear DNA. The exogenous DNA was internalized via direct DNA uptake. The mechanism of active DNA import was independent of DNA sequence and involved the voltage-dependant anion channel (VDAC) translocase of the outer mitochondrial membrane (Tom20 and Tom40), specific for import of proteins and tRNA[9,10]. The imported DNA was integrated into the mitochondrial genome through homologous recombination[11-13]. If the exogenous DNA contains a gene controlled by a mitochondrial promoter, the DNA can be transcribed and processed into mature RNA molecules to form functional proteins in the mitochondria[11,13,14].

An alternative approach to genetic engineering of plant mitochondria that can be applied to intact plant cells is based on mitochondrial targeting peptides (mTPs). The experimental criteria applied to identify mTPs emulate the existence of a protein sorting signal with similar physicochemical properties as a cell penetrating peptide (CPP) called Transactivator of Transcription (Tat). Cell penetrating peptides are defined as short cationic peptides which are capable of transducing cargo varying in size, chemical properties, or function across cell membranes in a receptor independent manner[15]. Tat's cell penetrating properties are derived from the peptides' positive charge and the guanidinium head group of the arginine residues which facilitate electrostatic membrane interaction followed by translocation of the peptide and its cargo through the plasma membrane[16]. Tat and its attached cargo accumulate in the nucleus of transfected cells due to the presence of a protein sorting signal called a nuclear localization signal (NLS) within its peptide sequence[17].

Mitochondrial proteins transcribed from nuclear DNA are translated in the cytosol and targeted to the mitochondria within the cell by short peptide sequences on the N-terminus, C-terminus and/or contained within the protein sequence[18]. These short peptide sequences are referred to as pre-sequences and mitochondrial or matrix targeting peptide (mTPs) sequences for mitochondrial proteins[19,20]. Mitochondrial translocases of the outer/inner membrane (TOM/TIM) bind to protein mTPs and facilitate the transport of the nascent cytosolic proteins across the

double membrane of mitochondria[21]. In addition, mTPs were found to be able to transport other cargoes into cell mitochondria, including cancer drugs[22,23] and RNAs[24].

Organelle (chloroplast and mitochondria) genome transformation is associated with a challenge of high number of organelles per cell and high number of genomes per organelle. In chloroplast transformation, generation of transplastomic plants with genetically uniform, homogeneous chloroplast genomes (homoplastomic plants) can be achieved by means of discriminatory selection that favors transgenic copies of ptDNA over the unmodified genomes, and maintains them during replication and sorting[25]. Application of a selection agent during post-transfection tissue culture enables enrichment of chloroplasts with genetically engineered genomes. Therefore, efficient selection and regeneration procedures are needed for successful generation of transformed plants. Similar rules are expected to apply also for generation of homoplasmic plants with stably transformed mitochondrial genomes. Cells that survive selection will contain only a subset of transfected mitochondria out of the entire population of mitochondria in the cell. Therefore, a selectable marker is required to select for mitochondria expressing the selectable marker gene while eliminating mitochondria that have not taken up the DNA construct.

The most common selectable marker used for organelle transformation is the bacterial *aadA* gene, which encodes aminoglycoside 3` adenyl transferase and confers high levels of resistance to spectinomycin and streptomycin[26]. However, the selection was for the contrast between green tissue and chlorotic tissue rather than for survival and growth[27]. The *aadA* gene was used initially as a marker for transformation of nuclear genomes[27]. Soon after, *aadA* was successfully employed as a marker for transformation of plastids[28]. In plastid transformation, the dominant *aadA* selectable marker gene replaced the hitherto used recessive marker which also conferred resistance to spectinomycin but originated from the plastid 16S rRNA gene[28].

In addition to selectable markers, reporter genes are useful tools to monitor transformation events by imaging expression of the delivered genes. The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was reported to function as a sensitive and nondestructive reporter of *in vivo* gene expression[29,30]. The wild-type GFP has been used for expression studies in plants in various transformation systems[31]. Mutants of GFP with increased stability and enhanced fluorescence have been isolated to improve detection[31-33]. Moreover, the use of fusion proteins where the coding region of a reporter gene is joined in-frame with a second gene of interest has been particularly useful. Fusion of the *gfp* reporter gene and the *aadA* selectable marker gene (*aadA:gfp*) allows for visual deletion of gene expression through green fluorescence of GFP as well as for selection of transfected cells through resistance to antibiotics (dual selection)[34].

Our protocol presents a new method for gene delivery into the mitochondria of intact plant cells. The method is based on mTP peptide nano-carriers, which combine cell penetrating and mitochondria targeting properties. To demonstrate the versatility of the mTP-DNA protocol, we tested it in both somatic and germ cell systems and showed binding and delivery of a linear double-stranded DNA reporter cassette (*aadA:gfp*) into the mitochondria of triticale protoplasts

and microspores, followed by documented expression in organello. Furthermore, we demonstrated that plantlets with exogenous DNA integrated into the mitochondrial genome can be regenerated from microspores transfected with *aadA:gfp* DNA.

PROTOCOL:

1. Preparation of culture media and cells

1.1. Stock solutions

1.1.1. NPB-99 Macro-salts (10x). Prepare 1 L of 10x Macro-salts stock solution by dissolving 14.15 g of KNO_3, 2.32 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, 0.83 g of $CaCl_2{\cdot}2H_2O$ and 0.93 g of $MgSO_4{\cdot}7H_2O$ in 500 mL of ddH_2O. Stir the solution at room temperature and bring the volume to 1 L with ddH_2O.

1.1.2. NPB-99 Micro-salts (100x). Prepare 1 L of 100x Micro-salts stock solution by dissolving 40 mg of KI, 0.5 g of $MnSO_4{\cdot}7H_2O$, 0.5 g of H_3BO_3, 0.5 g of $ZnSO_4{\cdot}7H_2O$, 1.25 mL of 1 mg/mL $CoCl_2{\cdot}6H2O$, 1.25 mL of 1 mg/mL $CuSO_4{\cdot}5H_2O$ and 1.25 mL of 1 mg/mL $Na_2MoO_4{\cdot}2H_2O$ in 500 mL of ddH_2O. Stir the solution at room temperature and bring the volume to 1 L with ddH_2O.

1.1.3. Fe-EDTA (100x). Prepare 100 mL of 100x Fe-EDTA stock solution by dissolving 278 mg of $FeSO_4.7H_2O$ and 372 mg of Na_2EDTA in 50 mL of ddH_2O. Stir the solution at room temperature and bring the volume to 100 mL with ddH_2O.

1.1.4. NPB-99 vitamins (100x). Prepare 500 mL of 100x NPB-99 vitamins stock solution by dissolving 250 mg of thiamine-HCl, 25 mg of pyridoxine-HCl and 25 mg of nicotinic acid in 450 mL of ddH_2O. Stir the solution at room temperature and bring the volume to 500 mL with ddH_2O.

1.1.5. GEM Macro-salts (10x). Prepare 1 L of 10x Macro-salts stock solution by dissolving 19 g of KNO_3, 1.65 g of $(NH_4)NO_3$, 1.7 g of KH_2PO_4, 4.4 g of $CaCl_2{\cdot}2H_2O$ and 3.7 g of $MgSO_4{\cdot}7H_2O$ in 500 mL of ddH_2O. Stir the solution at room temperature and bring the volume to 1 L with ddH_2O.

1.1.6. GEM Micro-salts (100x). Prepare 1 L of 100x Micro-salts stock solution by dissolving 2.2 g of $MnSO_4{\cdot}7H_2O$, 0.62 g of H_3BO_3, 0.86 g of$ZnSO_4{\cdot}7H_2O$, 20 mL of 1 mg/mL $CoCl_2{\cdot}6H_2O$, 15 mL of 1 mg/mL $CuSO_4{\cdot}5H_2O$ and 25 mL of 1 mg/mL $Na_2MoO_4{\cdot}2H_2O$ in 500 mL of ddH_2O. Stir the solution at room temperature and bring the volume to 1 L with ddH_2O.

1.1.7. GEM vitamins (100x). Prepare 1 L of 100x GEM vitamins stock solution by dissolving 100 mg of thiamine-HCl, 100 mg of pyridoxine-HCl, 100 mg of nicotinic acid, 40 mg of ascorbic acid, 50 mg of pantothenate, 20 mg of riboflavin, 20 mg of folic acid, 20 mg of biotin, 790 mg of betaine chloride and 1000 mg of choline-HCl in 500 mL of ddH_2O. Bring the volume to 1000 mL with ddH_2O.

NOTE: Filter sterilize the stocks solutions 100x NPB-99Macro-salts (step 1.1.1), 100x NPB-99

Micro-salts (step 1.1.2), 100x Fe-EDTA (step 1.1.3), 10x GEM Macro-salts (step 1.1.5) and 100x GEM Micro-salts (step 1.1.6) using a 0.22 μm filter and store at 4 °C. Filter sterilize the 100x vitamin stock solutions (steps 1.1.4 and 1.1.7) using a 0.22 μm filter, aliquot 10 mL into 15 mL plastic tubes and store at -20 °C.

1.2. Solutions and media

1.2.1. NPB-99 extraction and wash solution

1.2.1.1. Prepare 1 L of NPB-99 extraction and wash solution by starting with 500 mL of ddH_2O in a 2 L beaker on a stir plate and adding 100 mL of the 100x NPB-99Macro-salts stock solution, 10 mL of the 100x NPB-99 Micro-salts stock solution, 10 mL of the 100x Fe-EDTA stock solution, 10 mL of the 100x NPB-99 vitamins stock solution.

1.2.1.2. Supplement the solution with 50 mg of myo-inositol, 500 mg of L-glutamine, 90 g of maltose monohydrate, 200 μL of 1 mg/mL 2,4-D stock solution, 200 μL of 1 mg/mL kinetin and 1 mL of 1 mg/mL PAA.

1.2.1.3. Bring volume to 1 L with ddH_2O and adjust pH to 7.0.

1.2.2. NPB-99 culture medium

1.2.2.1. Prepare 200 mL of NPB-99 culture medium by starting with 100 mL of ddH_2O in a 400 mL beaker on a stir plate and adding 20 mL of the 100x NPB-99Macro- salts stock solution, 2 mL of the 100x NPB-99 Micro-salts stock solution, 2 mL of the 100x Fe-EDTA stock solution, 2 mL of the 100x NPB-99 vitamins stock solution.

1.2.2.2. Supplement the solution with 10 mg of myo-inositol, 100 mg of L-glutamine, add 200 μL of the 10 mg/mL arabinogalactan solution, 18 g of maltose monohydrate, 20 g of ficoll, 40 μL of 1 mg/mL 2,4-D solution, 40 μL of 1 mg/mL kinetin and 200 μL of 1 mg/mL PAA.

1.2.2.3. Bring volume to 200 mL with ddH_2O and adjust pH to 7.0.

1.2.3. GEM Medium

1.2.3.1. To prepare 2 L of GEM medium, first make 1 L of 0.6% (w/v) solidifying agent 1 solution by adding 6.0 g of solidifying agent 1 and a large magnetic stir bar to 1 L of ddH_2O in a large (2 or 3 L) Erlenmeyer flask and stirring for 5 min to disperse solidifying agent 1, and then autoclave for 20 min and cool to 65 °C.

1.2.3.2. Make 2x GEM medium solution by adding to 500 mL of ddH_2O in a 2000 mL beaker, 200 mL of the 10x GEM Macro-salts stock solution, 20 mL of the 100x GEM Micro-salts stock solution, and 20 mL of the 100x Fe-EDTA stock solution.

1.2.3.3. Supplement the 2x GEM solution with 30 g of maltose monohydrate, 10 g of sucrose, 0.7 g of xylose, 0.7 g of ribose, 0.4 g of myo-inositol, 0.71 g of U2.5 Amino Acid Mixture, 1.5 g of L-glutamine, 2 mg of spermine, 8 mg of spermidine and 20 mL of 100x GEM vitamins stock solution.

1.2.3.4. Add soluble organic acids: 2.0 g of malic acid, 0.4 g of fumeric acid, 0.04 g of succinic acid, 0.04 g of α-ketoglutaric acid, 0.01 g of pyruvic acid, 0.01 g of citric acid and 2 mL of plant preservative mixture. Bring volume to 1 L with ddH_2O and adjust pH to 5.8 with KOH. Filter sterilize the 2x GEM medium solution using a 0.22 μm filter.

1.2.3.5. Mix the 2x GEM medium with 0.6% solidifying agent 1 solution by continuous stirring on a hot stir plate. Add 8 mL of streptomycin solution (50 mg/mL; final concentration: 200 mg/mL), mix and aliquot 25 mL medium into 9 cm Petri dishes; once solidified, store plates at 4 °C.

NOTE: Avoid melting solidified agent 1 and GEM medium: solidified and then re-melted agent 1 will not re-solidify.

1.2.4. **Rooting Medium**

1.2.4.1. Prepare 1 L of rooting medium by starting with 500 mL of ddH_2O and adding 100 mL of the 10x GEM Macro-salts stock solution, 10 mL of the 100x GEM Micro-salts stock solution, 10 mL of the 100x Fe-EDTA stock solution, 1.48 g of NH_4NO_3, 0.83 mL of 1 mg /mL KI and 10 g of sucrose. Bring volume to 1 L with ddH_2O and adjust pH to 6.0 with 1 N KOH.

1.2.4.2. Transfer the solution into a 2 L bottle and add 6 g of agar. Autoclave for 15 min and cool to 65 °C.

1.2.4.3. Add 0.5 mL of filter sterilized PAA (1 mg/mL) solution and 8 mL of streptomycin solution (50 mg/mL; final concentration: 400 mg/mL), mix and aliquot the rooting medium into 250 mL magenta vessels (50 mL) or 15 mL glass tubes (5 mL). Once medium is solidified, store it at 4 °C.

1.3. Preparation of plant cells

1.3.1. Isolate microspore cells at the mid-late uninucleate stage from the surface-sterilized anthers of triticale (*X. Triticosecale* Wittmack, cv. Ultima)[35] according to the previously published protocol[36].

1.3.2. Isolate and purify protoplasts from Triticale leaves by following the protocol published previously[37].

1.3.3. Adjust the final cell concentration to 2 x 10^5 cells/mL (500 μL = 100,000 cells) with the NBP-99 medium (microspores) or CPW solution (protoplasts)[37].

NOTE: Other triticale cultivars as well as other plant species (e.g., wheat can be used as a source of cells for transfection).

2. Preparation of DNA and peptides

2.1. Construction of a vector carrying a reporter gene construct for mitochondrial expression

NOTE: The wheat mitochondrial *aadA:gfp* reporter cassette (*aadA:gfp*, **Figure 1A**) is a 3424 base pair long plant mitochondrial transfection vector designed to target insertions in between the *trnfM-1* and *rrn18-1* genes of the triticale (*X Triticosecale* Wittmack) and wheat (*Triticum aestivum)* mitochondrial genome.

2.1.1. Design the vector using a bioinformatics software.

2.1.2. Start with the nucleotide sequence 300379-300878 (GenBank accession No. AP008982.1) as the left sequence flanking the cassette for its integration into the mitochondrial genome via homologous recombination (5' HR) with the *trnfM-1* target insertion sequence.

2.1.3. Introduce a multiple cloning site following the *trnfM-1* target insertion sequence.

2.1.4. Add the selection marker gene cassette sequence: (i) the *T. aestivum* mitochondrial *atpA* gene promoter (P_{atpA}; GenBank accession No. X54387.1), (ii) *aadA:gfp* fusion gene[34] (GenBank accession No. ABX39486) and (iii) the T_{cobA} terminator (GenBank accession No. AP008982.1, nucleotides: 62871-62565).

NOTE: Other selectable marker genes and mitochondrial gene expression elements (promoters and terminators) may be used.

2.1.5. End the design with the nucleotide sequence 300880-301373 (GenBank accession No. AP008982.1) as the right sequence flanking the cassette for its integration into the mitochondrial genome via homologous recombination (3' HR) with the *rrn18-1* target insertion sequence.

2.1.6. Order synthesis of the designed reporter gene construct by a DNA synthesis service. The construct will be delivered as a bacterial plasmid vector containing the reporter gene cassette (e.g., pMK_Wheat_Mito_aadA-gfp).

2.1.7. Perform plasmid preparation following the manufacturer protocol and using the kits summarized in the **Table of Materials**.

2.2. DNA cargo preparation

2.2.1. Linearize plasmid DNA by digestion with a single-cutter restriction enzyme (e.g., *Pst*I) according to the supplier's protocol.

2.2.2. Purify the linearized plasmid DNA following the manufacturer protocol and using the kit summarized in the **Table of Materials**.

NOTE: Good quality of DNA is crucial for transfection.

2.2.3. Dilute DNA in nuclease-free/protease-free H_2O to obtain 150 ng/µL stock solution.

2.2.4. Per each transfection, aliquot 10 µL of the 150 ng/µL DNA stock solution (total DNA amount: 1.5 µg) into sterile 1.5 mL tubes.

2.2.5. Add 40 µL of nuclease-free/protease-free H_2O into each tube (final volume: 50 µL) and mix the tube content by gentle pipetting. Scale up for multiple transfections by preparing the DNA working stock solution in a volume proportional to the number of transfections.

2.3. mTP peptide carrier preparation

2.3.1. Order synthesis of the mTPs by peptide synthesis service. The peptide will be delivered as white powder, which should be stored at -20 °C.

2.3.2. Prepare 1 mg/mL peptide stock by dissolving 1 mg of the peptide powder in 1 mL of nuclease-free/protease-free H_2O. Aliquot 100 µL of the stock into sterile 0.6 mL tubes and store them at -20 °C until use.

2.3.3. Thaw the peptide stock on ice prior to transfection. Do not exceed three thawing-freezing cycles.

2.3.4. Prepare the working stock solution by aliquoting the nuclease-free/protease-free H_2O into sterile 1.5 mL tubes, adding the appropriate volume of the 1 mg/mL peptide stock (**Table 1**), and mix gently. Scale up for multiple transfections by preparing the peptide working stock solution in a volume proportional to the number of transfections.

3. Transfection of triticale microspores with mTP-DNA nano-complexes (Figure 1B)

3.1. Formation of mTP-DNA nano-complexes

3.1.1. Aliquot 50 µL of the DNA working stock solution into a sterile 1.5 mL tube and add 50 µL of the peptide working stock solution, mix gently.

3.1.2. Incubate the peptide-DNA mixture for 10 min at room temperature; mix gently by tapping the tube bottom every 5 min. Do not exceed 15 min, because prolonged incubation will result in aggregation of the peptide-DNA complexes.

3.2. Delivery of mTP-DNA nano-complexes into triticale microspores

3.2.1. Aliquot 500 µL of the 2 x 10^5 cells/mL microspore cell suspension (step 1.2; total 100,000

cells per transfection) into sterile 2 mL tubes.

3.2.2. Add 100 µL of the peptide-DNA mixture (step 2.1) to microspores and mix gently. Include control treatments by omitting the peptide or DNA component, or both.

3.2.3. Incubate the peptide-DNA-microspores transfection mixture for 1 h at room temperature; mix gently by tapping the tube bottom every 5 min.

3.2.4. Add 400 µL of the NPB-99 medium (step 1.1.1) and continue incubation at room temperature for 24 h (confocal microscopy) or 48 h (qRT-PCR) or 1 h (microspore culture and selection).

NOTE: Samples for confocal microscopy analysis should be incubated in the dark.

4. Nucleic acids extraction from the transfected cells and plant tissues

4.1. Harvest microspore cells by centrifugation, freeze the cell samples in liquid nitrogen and store at -80 °C until use.

4.2. Disrupt frozen cells with ceramic or metal bead by aggressive shaking in a cell/tissue disrupting machine (2 times 2 pulses for 60 s at 4,000 x *g*).

NOTE: Cells may be also disrupted by vortexing with metal beads in lysis buffer for 15 min at maximum speed (1,000 x *g*) at room temperature.

4.3. Isolate gDNA and RNA following the manufacturer protocol and using the kits summarized in the **Table of Materials**, with some modifications, including additional on-column DNase I digestion. Alternatively, the additional DNase I digestion can be done on the purified RNA samples, followed by clean up on column, according to the manufacturer protocol.

4.4. Asses the quantity and quality of DNA and RNA using agarose gel electrophoresis (to ensure DNA and RNA integrity) and spectrophotometrically (to quantify the DNA and RNA).

5. Analysis of transient expression of the delivered transgene

5.1. Real-time RT-PCR (qRT-PCR)

5.1.1. Generate cDNA from 1 µg of total RNA isolated from transfected and control cells following the manufacturer protocol and using the kits summarized in the **Table of Materials**.

5.1.2. Perform real-time RT-PCR according to protocol outlined in **Table 2,** using GFP1L/GFP1R (for *aadA::gfp*) and EF_F1/EF_R1 (for *EF1α*) primers (**Table 3**). Calculate relative expression of the *aadA::gfp* gene in relation to the expression of the endogenous *EF1α* gene, using the standard curve method.

5.2. Confocal microscopy

5.2.1. Add fluorescent dye (3.0 µL of 1.0 mM mitochondria-specific dye; final concentration: 3 µM) to 1 mL of transfected cells 24-48 h after transfection to stain cell mitochondria.

5.2.2. Observe cells using a confocal microscope to examine the subcellular localization the GFP protein (excitation/emission wavelength: 490 nm/520 nm) by collecting fluorescence emissions in z-confocal planes of 10–15 nm and analyzing the images using appropriate software. Compare it to localization of the cell mitochondria stained with mitochondria-specific dye (excitation/emission wavelength: 554 nm/576 nm).

6. Selection and regeneration of putative transformants

6.1. Microspore embryogenesis

6.1.1. Incubate the transfected and control microspores for 1 h at room temperature and then transfer to 35 mm Petri dishes containing 2.5 mL of NBP-99 medium (total volume: 3.5 mL) and 4 ovaries. Add streptomycin (7 µL of 50 mg/mL stock; final concentration: 100 mg/L).

6.1.2. Seal 35 mm plates with semi-transparent flexible film and place them in a 150 mm Petri dish with 60 mm dish containing distilled water. Seal the 150 mm dish with semi-transparent flexible film to keep moisture in.

6.1.3. Culture the cells in the dark at 27 °C to induce embryogenesis. Carry microspore control cultures with or without streptomycin to determine the effect of antibiotics on microspore embryogenesis and green plant regeneration.

6.2. Embryo germination

6.2.1. After 4 weeks of culture, transfer the developing embryos onto GEM plates supplemented with streptomycin (final concentration: 200 mg/L).

6.2.2. Culture embryos at 16 °C beneath wide spectrum 40 watts bulbs delivering 80 µM m^{-2} s^{-1} (a 16-h light period) for embryo germination.

NOTE: Only a few embryos will germinate into green plantlets. Some embryos will develop into albino plants or may form roots only, while the majority of embryos will be aborted.

6.3. Rooting of green plantlets

6.3.1. After 3 to 4 weeks, transfer green plantlets into magenta vessels containing the rooting medium with streptomycin (final concentration: 400 mg/L) and continue plantlet cultivation as described above (step 6.2.2).

NOTE: This selection procedure results in regeneration of haploid putative *aadA:gfp* plants. In order to obtain fertile, doubled haploid plants, haploid plants may be treated with colchicine to induce chromosome duplication[38,39] or cultivated in soil for spontaneous genome duplication.

7. PCR screening of putative transformants

7.1. gDNA isolation

7.1.1. Collect about 100 mg of leaf samples from the regenerated plants, freeze the tissue samples in liquid nitrogen and store at -80 °C until use.

7.1.2. Disrupt frozen cells with ceramic or metal beads by shaking aggressively in a cell/tissue disrupting machine (2 times 2 pulses for 60 s at 4,000 x *g*) or by crushing leaf tissue with a pestle in tubes frozen in liquid nitrogen.

7.1.3. Isolate genomic DNA from young leaves of the primary transformants (T_0) at the end of in vitro culture steps using a kit summarized in the **Table of Materials**, following the manufacturer's protocol.

7.2. PCR

7.2.1. Perform PCR using GFP1L/GFP1R primers (**Table 3**) according to the protocol outlined in **Table 4**.

7.2.2. Separate the PCR samples by electrophoresis in a 1.0% agarose gel containing ethidium bromide in 1x TAE buffer for 1 h.

8. Analysis of transgene insertion into mitochondrial genome

8.1. Amplification and cloning of the left and right junctions

8.1.1. Perform PCR according to protocol outlined in **Table 4** by using 3 sets of primers for each junction. Left junction primer sets: trnfMF3/trnfMR2, trnfMF6/trnfMR4 and trnfMF5/trnfMR4; right junction primer sets: rrn18-1F3/rrn18-1R4, rrn18-1F3/rrn18-1R6 and rrn18-1F4/rrn18-1R5 (**Table 3**).

8.1.2. Separate the PCR samples by electrophoresis in a 1.0% agarose gel containing ethidium bromide in 1x TAE buffer for 1 h.

8.1.3. Purify the PCR products using a gel extraction kit (**Table of Materials**).

8.1.4. Clone the PCR products into a PCR cloning vector (**Table of Materials**), according to the manufacturer's protocol.

NOTE: Other PCR cloning kits may be used.

8.2. Sequencing and sequence alignment

8.2.1. Order sequencing of the cloned PCR products through a DNA sequencing service, using the universal M13 and T7 primers.

8.2.2. Conduct sequence alignments using a bioinformatics software as described previously[37]. Use pairwise or multiple alignment to generate the consensus sequence for each junction. Then, apply multiple genome alignment using *aadA::gfp* construct sequence with 500 bp extensions of the 5' and 3' HR (homology regions) as a target (reference) sequence to analyze nucleotide sequence similarity between the consensus junction sequences and the reference sequence.

REPRESENTATIVE RESULTS

DNA binding and protection properties of mTPs

The ability of mTPs to non-covalently bind nucleic acids is an essential property for translocation of dsDNA cargoes into the plant mitochondria. The minimum peptide amount needed to bind to and completely saturate the linearized dsDNA in preparation for microspore transfection was determined in a gel mobility shift assay and a nuclease protection assay. Titration of the mTP peptide was performed by adding increasing amounts of the peptide to a small, constant quantity of linearized plasmid DNA. All five mTPs caused a shift in the DNA mobility during electrophoresis (**Table 5**). **Figure 2A** presents results of the titration experiment for the mTP1 peptide. Analogous results for other mTPs were published previously[37]. A complete shift in DNA mobility caused by the peptides was observed at the weigh-to-weight peptide:DNA binding ratio ranging from 4 to 6 μg of peptide per 1 μg of DNA. In addition, the nuclease protection assay showed that all five mTPs were able to protect DNA from nuclease degradation (**Table 5**, **Figure 2B** for mTP1) at the peptide:DNA ratios ranging from 8 to 25 μg of peptide per 1 μg of DNA. These ratios are about 2-4 folds higher than those required for a gel shift (**Table 5**).

Cell transfection and transient expression of mTP-delivered gene in triticale cells

mTPs were used to transfect somatic and germ cells of triticale plants with the *aadA:gfp* reporter DNA (**Figure 1A**) by exposing cells to pre-formed mTP-DNA nano-complexes (**Figure 1B**). The amount of peptides used for formation of the complexes was four times higher than the amount needed to bind the DNA. It was sufficient to protect DNA from nucleolytic degradation (**Table 5**), thus ensuring the integrity of the delivered DNA in plant cells. Two transient expression assays were used to test the mTP-mediated delivery of the reporter gene construct into mitochondria of triticale somatic cells (protoplasts of leaf mesophyll) and germ cells (microspores) (**Figure 3**). Production of the GFP protein in transfected protoplasts was analyzed by confocal microscopy and compared to the distribution of a mitochondria-specific orange fluorescence dye. Both green fluorescence (GFP) and orange fluorescence (dye) was detected in *aadA:gfp*-transfected protoplasts, and the fluorescence signals were observed to co-localize (**Figure 3A**), indicating that the GFP protein was produced in mitochondria of transfected protoplasts. Second, production of *aadA:gfp* transcripts was quantified by real-time RT-PCR performed 48 h after transfection of

triticale protoplasts and microspore cells. The quantity of the *aadA:gfp* transcript in protoplasts ranged between 32 and 159 fold as compared to the house keeping control [*Elongation Factor 1α* (*EF1α*)], whereas the *aadA:gfp* transcript levels were lower in microspores (**Figure 3B,C**).

Regeneration and molecular screening of putative transformants

Microspores transfected with the *aadA:gfp* reporter construct DNA by mTP1 were subjected to streptomycin selection upon regeneration into embryos, green plantlets and green rooted plants (**Figure 4A**). From 28 transfection reactions a total of ten microspore derived green rooted plants were regenerated with increasing streptomycin selection pressure, and were named lines WM-aadAGFP-01 through 10. Control plants were regenerated more efficiently without streptomycin selection, although many embryos germinated into albino plantlets. Control microspores subjected to streptomycin selection regenerated two escape plants. The genomic DNA was isolated from ten plants regenerated from transfection experiments and from two control plants. Next, the standard (end-point) PCR was performed in order to identify plants carrying the *aadA::gfp* reporter gene. The production of an amplicon of a correct size was found in eight out of the 10 selected plants, while plants regenerated from control microspores did not show any PCR product (**Figure 4B**). Eight PCR-positive transformants were regenerated from total 2.8 million microspore cells used for 28 transfections; thus the method efficiency is about 3 transgenic plant per one million starter cells or 0.3 plants per transfection.

Identification of the *aadA::gfp* transgene insertion site

Eight PCR-positive WM-aadAGFP lines were analyzed for the *aadA:gfp* gene insertion into the mitochondrial *trnfM – rrn18-1* region. In order to determine the nucleotide sequence of the insertion, PCR was performed to amplify junctions at both ends of the *aadA::gfp* reporter cassette with the mitochondrial genomic DNA (left and right junctions; **Figure 5**). Primers specific to the wheat mitochondrial *trnfM – rrn18-1* region and to the *aadA* and *gfp* genes in the *aadA:gfp* construct were used for junction amplification (**Figure 5A**). Amplicons of the expected size were produced in the eight selected plants (**Figure 5B** presents data for the line # WM-aadAGFP-07). No PCR amplicons were produced in either of the two escaped control plants regenerated in the presence of streptomycin. The PCR products were cloned into a PCR cloning vector and sequenced. These sequences were then applied for reconstruction of the integration site in the *aadA:gfp* gene construct into the *trnfM – rrn18-1* region of triticale mitochondrial genome (**Figure 5C,D**). The amplified left and right junction sequences were aligned to the mitochondrial genomic target sequence and to the gene construct. The alignment analysis demonstrated insertion of the *aadA:gfp* cassette into the target region in six plants (lines # WM-aadAGFP-04 and WM-aadAGFP-06 through 10). **Figure 6** and **Figure 7** present sequence alignment data for the left and right junction, respectively, in the WM-aadAGFP-07 line. The quality of the sequencing data for two other plants (WM-aadAGFPWM-03 and WN-aadAGFP-05) was insufficient for sequence alignment analysis. Importantly, alignment of the assessabled left and right junction sequences in the WM-aadAGFP plant lines to the reference sequence mimicking insertion of the *aadA:gfp* cassette into the mitochondrial target site (*aadA::gfp* construct with 500 bp extensions of the *trnfM-rrn18-1* region; **Figure 5C**) showed 100% sequence identity (**Figure 6** and **Figure 7**), thus confirming precise integration of the reporter gene cassette into the target site in the plant mitochondrial genome (**Figure 5D**). Hence, the mTP-based method

allowed for delivery of the reporter gene cassette, followed by its transient expression and integration into the mitochondrial genome of the regenerated plantlets.

FIGURE LEGENDS

Figure 1: Microspore transfection with mTP-DNA nano-complexes. (A) Genetic map of the *aadA:gfp* construct. Annotations: 5' HR site (sequence containing the wheat mitochondrial *trnfM-1* gene); MCS (the multiple cloning site); atpA Promoter (the wheat mitochondrial *atpA* gene promoter); *aadA* (the aminoglycoside-3'-adenylyltransferase spectinomycin/streptomycin resistance gene); Linker (the 48 bp (16 aa) linker between the *aadA* and *gfp* genes); GFP (the green fluorescence protein (*gfp*) gene); cobA Terminator (the rice *cobA* gene terminator); 3' HR site (sequence from the wheat mitochondrial *rrn18-1* gene); GFP1L and GFP1R (the GFP primer binding sites and amplicon representation). This figure has been modified from MacMillan et al. 2019[37]. (**B**) Workflow for triticale cell transfection. The steps for transfection are listed as follows: formation of the mTP-DNA nano-complexes, aliquoting the isolated microspore suspension, transfection by mixing the mTP-DNA nano-complexes with the microspores, incubation and addition of the NPB-99 medium. The next, alternative steps are indicated by arrow patterns: qRT-PCR analysis of transient *aadA::gfp* gene expression in transfected microspores (stripes), confocal microscopy analysis of GFP production in mitochondria of transfected cells (grid), microspore culture upon streptomycin selection (dots).

Figure 2: DNA binding and protection properties of mTP1. (**A**) Electrophoretic mobility shift of plasmid DNA in mTP1 complexes formed at various weight-to-weight (w:w) peptide:DNA ratios: 0:1, 0.2:1, 0.45:1, 0.9:1, 1.8:1, 3.6:1, 7.2:1, 14.4:1, 28.8:1. A complete shift was observed for ratios ≥3.6:1 (asterisk). (**B**) DNA protection by mTP1. Peptide-DNA complexes formed at various w:w ratios of mTP and linearized plasmid DNA (pDNA) were treated with DNase I nuclease. Lane 1: pDNA (Control, no DNase I). Lanes 2-7: mTP-pDNA complexes at 0:1, 8:1, 16:1, 24:1, 32:1, 43:1 w:w ratios, plus DNase I. Lane L: DNA size marker. A complete protection was observed at peptide:DNA ratios ≥16:1. This figure has been modified from MacMillan et al. 2019[37].

Figure 3: Expression of the *aadA:gfp* reporter cassette in triticale cells after DNA delivery by mTPs. (**A**) Production and localization of the GFP protein in triticale protoplasts transfected with the *aadA:gfp* gene construct were analyzed by confocal microscopy 24 h after transfection with mTP3. Left panel: the green channel showing discrete GFP fluorescence within a protoplast mediated by mTP3. Center panel: the orange channel showing fluorescence of the mitochondria specific dye. Right panel: a composite image of orange and green channels to show the co-localization. Scale bar: 5 μm. (**B, C**) *GFP* transcript abundance was analyzed in triticale microspores (**B**) and protoplasts (**C**) by quantitative real-time RT-PCR performed 48 h after transfection with mTP-DNA complexes. Three repetitions were used to measure the fold difference of normalized *GFP* mRNA expression compared to the control levels of *EF1α* mRNA expression. Data displayed are means ± s.e.; n = 3. NC, negative control. This figure has been modified from MacMillan et al. 2019[37].

Figure 4: Regeneration and screening of putative *aadA::gfp* transformants.(**A**) Workflow of the selection and regeneration procedure. The steps for selection/regeneration are listed as follows.

The transfected and control microspores were cultured in induction/selection medium (NPB-99 + 100 mg/L streptomycin) for 4-6 weeks until 1-2 mm long embryos are formed. Developed embryos were transferred onto the GEM medium with 200 mg/L streptomycin and cultured for 3-4 weeks to allow for embryo germination. Green plantlets were then transferred onto rooting medium with 400 mg/L streptomycin and cultivated for 2-4 weeks to produce green plants with strong root system. (**B**) PCR screening of putative transformants. The presence of mTP-delivered *aadA::gfp* reporter cassette in leaf tissue of putative transformants (plant lines WM-aadAGFP-01 through 10) was tested by PCR using primers specific for the *gfp* gene (**Figure 1**). Lane 1: no template control (NTC); lane 2: plasmid *aadA:gfp* (PC); lane 3: WM-aadAGFP-01; lane 4: WM-aadAGFP-02; lane 5: WM-aadAGFP-03; lane 6: WM-aadAGFP-04; lane 7: WM-aadAGFP-05; lane 8: WM-aadAGFP-06; lane 9: WM-aadAGFP-07; lane 10: WM-aadAGFP-08; lane 11: WM-aadAGFP-09; lane 12: WM-aadAGFP-10; lanes 13 and14: plants regenerated from control microspores upon selection. This figure has been modified from MacMillan et al. 2019[37].

Figure 5: Analysis of transgene insertion into the mitochondrial genome. (**A**) A scheme illustrating the design for PCR amplification of the insertion site (left and right junctions) based on the reference sequence mimicking an insertion event into the target *trnfM – rrn18-1* region (*aadA:gfp* reporter construct with over 500 bp extensions of the 5' and 3' HR sites). All sequence annotations correspond to **Figure 1**. Primer binding sites are indicated above the reference sequence. The expected size and positions of the amplicons are indicated below the reference sequence. (**B**) PCR amplification of the left and right junctions in plant line WM-aadAGFP-07. Lanes 1, 4 and 7: amplification of the left junction products with three sets of PCR primers (trnfMF3/trnfMR2, trnfMF6/trnfMR4 and trnfMF5/trnfMR4); lanes 10, 13 and 16: right junction amplification products with three sets of PCR primers (rrn18-1F3/rrn18-1R4, rrn18-1F3/rrn18-1R6 and rrn18-1F4/rrn18-1R5); lanes 2, 5, 8, 11, 14 and 17: tests of the left and right junction amplification primers on *aadA:gfp* plasmid DNA; lanes 3, 6, 9, 12, 15 and 18: test of the left and right junction amplification primers on AC Ultima wild type genomic DNA; lanes 19, 20 and 21: no template controls (NTC). (**C**) Alignment of DNA sequences (from top to bottom): insertion reference sequence (*aadA:gfp* construct with extensions); wheat mitochondrial genomic sequence of the *trnfM – rrn18-1* region (294591 – 312591; GenBank accession No. AP008982.1) (Mt gDNA); left and right junction regions; *aadA:gfp* reporter construct. Pink arrowhead: *trnfM-1* gene; violet box: MCS; orange box: linker. (**D**) A schematic illustration of the *aadA::gfp* reporter cassette insertion of into the *trnfM – rrn18-1* region of the mitochondrial genome. Left panel: the *trnfM – rrn18-1* region before and after insertion (top and bottom maps). Right panel: diagram of the wheat/triticale mitochondrial genome (GenBank accession No. AP008982.1). This figure has been modified from MacMillan et al. 2019[37].

Figure 6: Alignment of the left junction sequence to the reference sequences. The following sequences were compared using a multiple alignment software: rrn18-1 (wheat mitochondrial genomic repeat region *trnfM – rrn18-1* sequence (294591 – 312591; GenBank accession No. AP008982.1); RP4\trnfM1\atpA: *aadA:gfp* reporter cassette with left and right boarder 500 bp homologous sequence extensions; Plant 7 LB Assembly consensus sequence: consensus sequence of the T0 transgenic plant WM-aadAGFP-07 amplified left junction region. This figure has been modified from MacMillan et al. 2019[37].

Figure 7: Alignment of the right junction sequence to the reference sequences. The following sequences were compared using a multiple alignment software: rrn18-1 (wheat mitochondrial genomic repeat region *trnfM – rrn18-1* sequence (294591 – 312591; GenBank accession No. AP008982.1); RP4\trnfM1\atpA: *aadA:gfp* reporter cassette with left and right boarder 500 bp homologous sequence extensions; 22- Assembly 2 consensus sequence: consensus sequence of the T0 transgenic plant WM-aadAGFP-07 amplified right junction region. This figure has been modified from MacMillan et al. 2019[37].

Table 1: Preparation of the peptide working solution for a single transfection

Table 2: PCR program and sample composition for the real-time RT-PCR analysis

Table 3: Primer sequences

Table 4: PCR program and sample composition for the end-point analysis

Table 5: Sequence and properties of mTPs

DISCUSSION

Genetic engineering of mitochondria is a key tool in plant biology and biotechnology but has been very limited in its utility so far due to restrictions in DNA delivery into mitochondrial organelles. Biolistics and electroporation have proven to be popular methods of gene delivery into eukaryotic and prokaryotic cells[40], but there are limitations when applying these technologies to in vivo plant mitochondrial genetic transformation. Frequently mitochondrial transformation rates are low[41] in spite of mitochondrial natural competence to take up nucleic acids. This could be due to the physical damage caused to the plant tissue being too severe for the transformed cells to survive. Mitochondrial targeting peptides provide a much more benign procedure, giving transfected cells a better chance of survival.

Reporter gene construct

Cells that survive transfection need to be subjected to selection in order to eliminate untransfected cells and to enrich the transfected cells in mitochondrial genomes that carry the delivered gene. Therefore, the presented protocol includes a reporter gene construct that contains a selectable marker gene (*aadA*), which confers resistance to antibiotics[26]. In addition, the *aadA* gene is fused with a visible reporter gene (*gfp*), thus allowing the observation of the in vivo expression of delivered DNA. The *aadA::gfp* gene fusion contains a linker that separates the *aadA* and *gfp* genes, permitting proper folding of each component of the fusion AADA-GFP protein produced from the delivered gene and ensuring proper protein function[34]. Expression of the *aadA::gfp* reporter gene in plant mitochondria is controlled by mitochondrial gene expression elements: the *Triticum aestivum* mitochondrial *atpA* gene promoter (P_{atpA}) and cobA gene terminator (T_{cobA}). These control elements are universal for both wheat and triticale because these species share cytoplasm, and thus mitochondria, since wheat is the maternal parent of triticale (*X. Triticosecale* Wittmack) produced from a crossing between wheat (*Triticum aestivum)*

and rye (*Secale cereal*)[42,43]. Additionally, it is essential that the reporter gene expression cassette is flanked by sequences originating from the target site of the wheat mitochondrial genome (region *trnfM – rrn18-1*) in order to facilitate targeted insertion. While 1-2 kb long homology regions are routinely used in chloroplast transformation[44,45], this protocol proved that homologous sequences as short as 0.5-0.6 kb were sufficient for successful integration of delivered DNA through homologous recombination. Nevertheless, longer homologous sequences (1.5-2.8 kb) may also be used for mitochondrial transformation, as reported recently[45]. The protocol can potentially be adapted for gene transfer into the cells of other plant species by replacing the wheat mitochondrial gene expression control elements and homologous sequences with sequences originating from the desired species.

Mitochondrial transfection

The protocol for transfection of triticale and wheat mitochondrial genomes includes three key components that are critical for successful gene delivery: (i) formation of the mTP-DNA complexes, (ii) plant cells, and (iii) transfection procedure. DNA delivery into plant cells is achieved through peptides with both the cell penetrating and mitochondria targeting properties. Formulation of the mTP-DNA complexes in this protocol is based on the DNA binding and protection features of mTPs. The peptide amounts used to produce complexes with DNA are sufficient not only to bind the desired amount of DNA but also to protect it from nucleolytic degradation, thus securing the integrity of the delivered DNA in plant cells prior to its integration into the mitochondrial genomes. Interestingly, similar amounts of other peptides with the DNA delivering properties (MTPs, MTP-CPPs) were shown to be required for saturation of DNA binding, although more efficient gene expression was observed in Arabidopsis leaves and seedlings transfected with peptide-DNA complexes formed with very low amounts of the peptides[7,45]. The peptide amounts applied in this protocol allowed for efficient expression of the delivered gene, and thus may be specific for the peptide used.

The second key element of the gene delivery procedure is the type of plant cells subjected to transfection. The protocol was proven to be applicable to both somatic cells (leaf protoplasts) and germ cells (microspores). Based on the universal nature of other peptides, especially the cell penetrating peptides[46] known to share many properties with mTPs, it is very likely that the protocol can be applied to other plant cells of the same or different species. Finally, the transfection procedure is designed for the DNA transfer to occur under conditions most favorable for cells. It includes incubation of the cells with the mTP-DNA nano-complexes in the medium specific for the cell type (e.g., NPB-99 for microspores, CPW solution for protoplasts), followed by supplementation of the transfection mixture with additional volume of the medium prior to further analyses and cell culture. The ratio between the nano-complex volume and the cell suspension volume may affect the efficiency of transfection, especially in the case of cell types that are more sensitive to environmental factors (e.g. protoplasts) as compared to the cells that are better protected by a strong cell wall (e.g., microspores).

Cell culture, selection and plantlet regeneration

Plantlets with *aadA::gfp* engineered mitochondrial genomes can be regenerated post transfection through in vitro culture under selection. Although plant regeneration from

protoplasts or other explants is commonly used in plant biotechnology, microspores offer an exceptional facilitation in plant recovery from a single cell. Microspores, when cultured in vitro in an induction medium with properly balanced phytohormones, produce embryos without the intermediate stages involving callus formation and/or somatic embryos[36,47,48]. Microspore derived embryos germinate to produce seedlings, which can be then cultured on a rooting medium to enhance root system prior to transfer into soil. These plantlets may then be subjected to the doubled haploid production procedure (not included in this protocol) for generation of fertile plants[38,39].

Another advantage of using microspores for transfection and plant regeneration is their response to streptomycin antibiotic allowing for easier and effective selection. Spectinomycin and streptomycin cause chlorosis, rather than cell death, when used on plant tissues or germinating seedlings[27]. However, triticale microspores are sensitive to streptomycin (data not shown) thus making the *aadA* gene a good selectable marker for microspore transfection. In addition, use of streptomycin instead of spectinomycin as a selection agent shortens the protocol for plant recovery. Previous applications of the *aadA* marker gene in genetic transformation of plant organelles comprised of regeneration of plants upon spectinomycin selection[28]. In putative transplastomic plants expression of the *aadA* gene introduced into plastid genomes may confer resistance to spectinomycin[28]. Alternatively, a spontaneous mutation of the plastid *rrn16* gene encoding small ribosomal RNA may result in spectinomycin resistance[28]. In order to distinguish transgenic *aadA* plants from mutants, spectinomycin-resistant plants need to be tested for resistance to streptomycin[28,44]. Direct application of streptomycin to microspore culture makes the selection procedure less complicated and less laborious. Further streptomycin selection during embryo germination results in small green plantlets originating from *aadA::gfp* transfected microspores, along with albino plantlets and aborted embryos. It should be noted that albinotic plants regenerate often from microspore-derived embryos[36,39,47], and these occurrences present a limitation of the plant regeneration protocol. In addition, the severity of streptomycin selection needs to be balanced between very stringent selection, which eliminates transfected cells, embryos and plantlets expressing the *aadA* selection marker gene at low levels, and too lenient selection that allows for selection leakage. The presented protocol comprises gradual selection from 100 to 400 mg/L streptomycin and resulted in eight transgenic plants along with two non-transgenic plants (escapes) regenerated from microspore transfection experiments. However, more stringent selection may result in lower regeneration efficiency.

mTP-mediated DNA delivery provides a method that will find multiple applications in plant biology and biotechnology. It could be used for investigation of mitochondria in vivo, in plant cells. Moreover, this technology may hold the key to improvement of vital physiological processes and traits crucial in plant breeding, such as rates of photosynthesis, assimilation of nitrogen and biomass production as well as accumulation of secondary metabolites and resistance to pathogens.

ACKNOWLEDGMENTS

The authors thank Bernie Genswein for his computational assistance as well as the Kovalchuk Laboratory, Eric Amundsen and Victoria Hodgson for their technical assistance in the lab. This

work was supported by Agriculture and Agri-Food Canada and Agriculture Funding Consortium (Alberta Innovates/Biosolutions, Alberta Wheat Commission and Saskatchewan Wheat Development Commission).

DISCLOSURES

The authors have no conflict of interest to disclose.

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Figure 1

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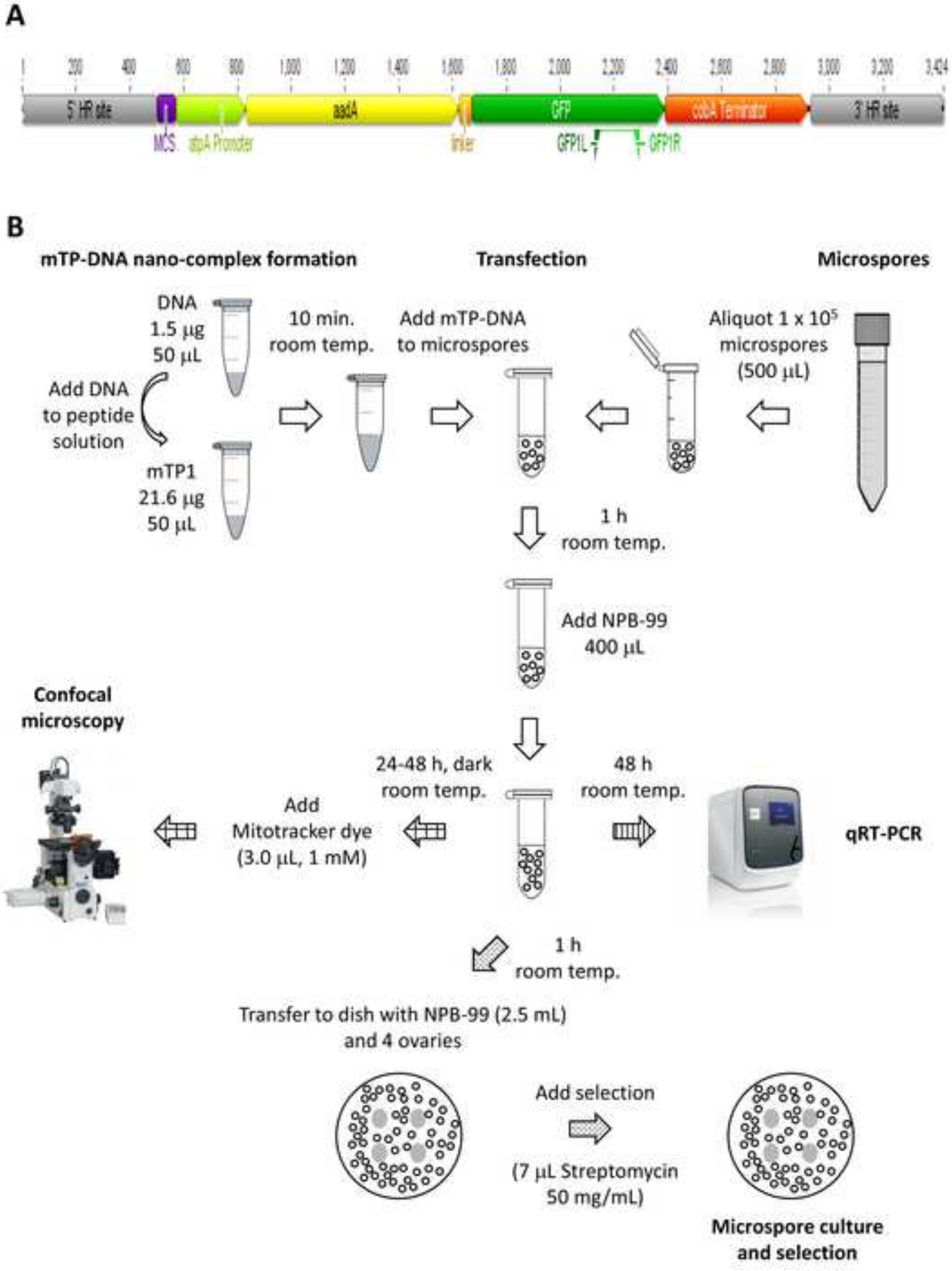


Figure 2

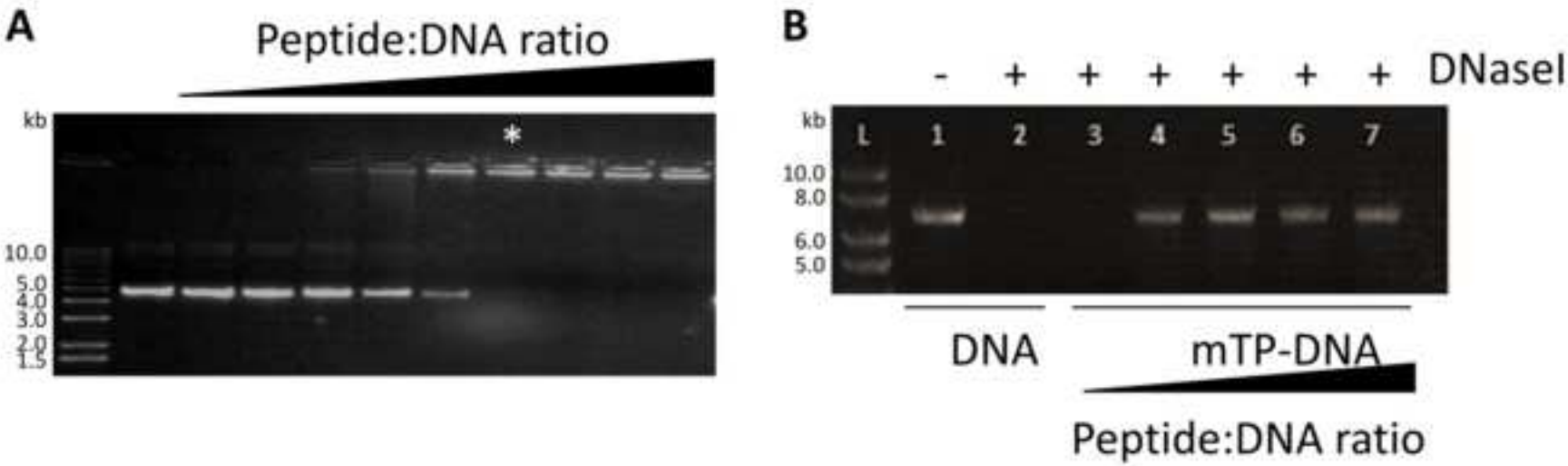


Figure 3

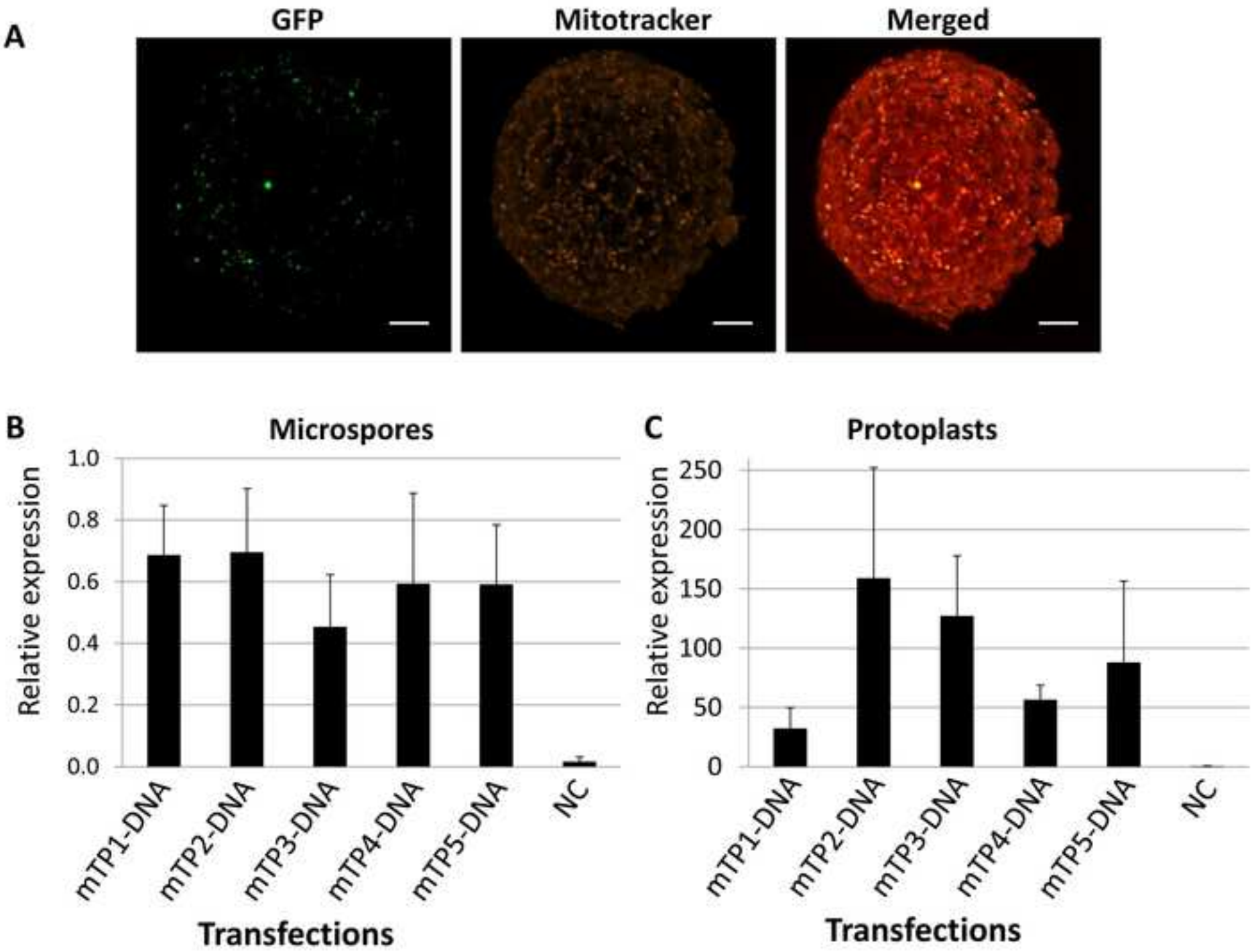
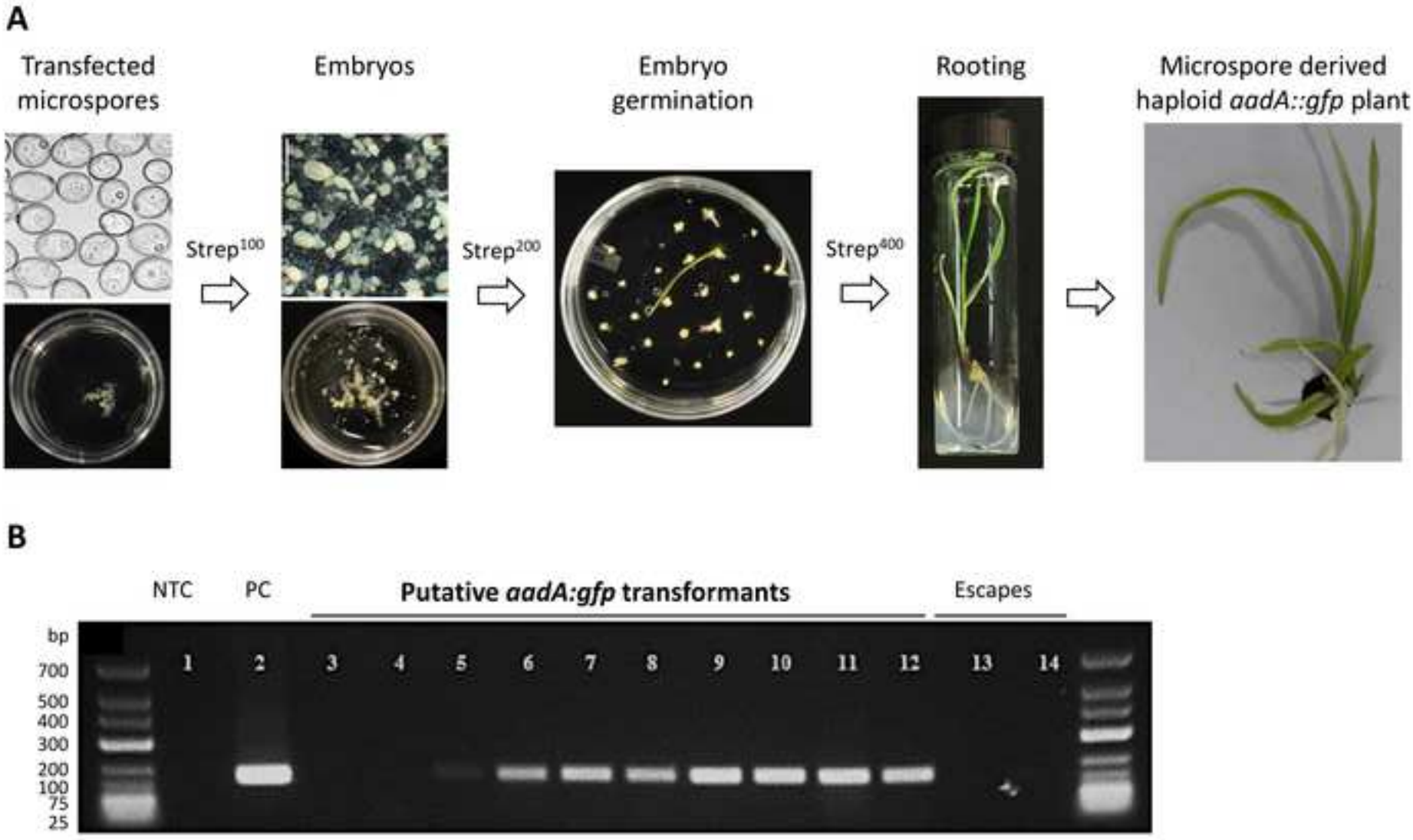


Figure 4



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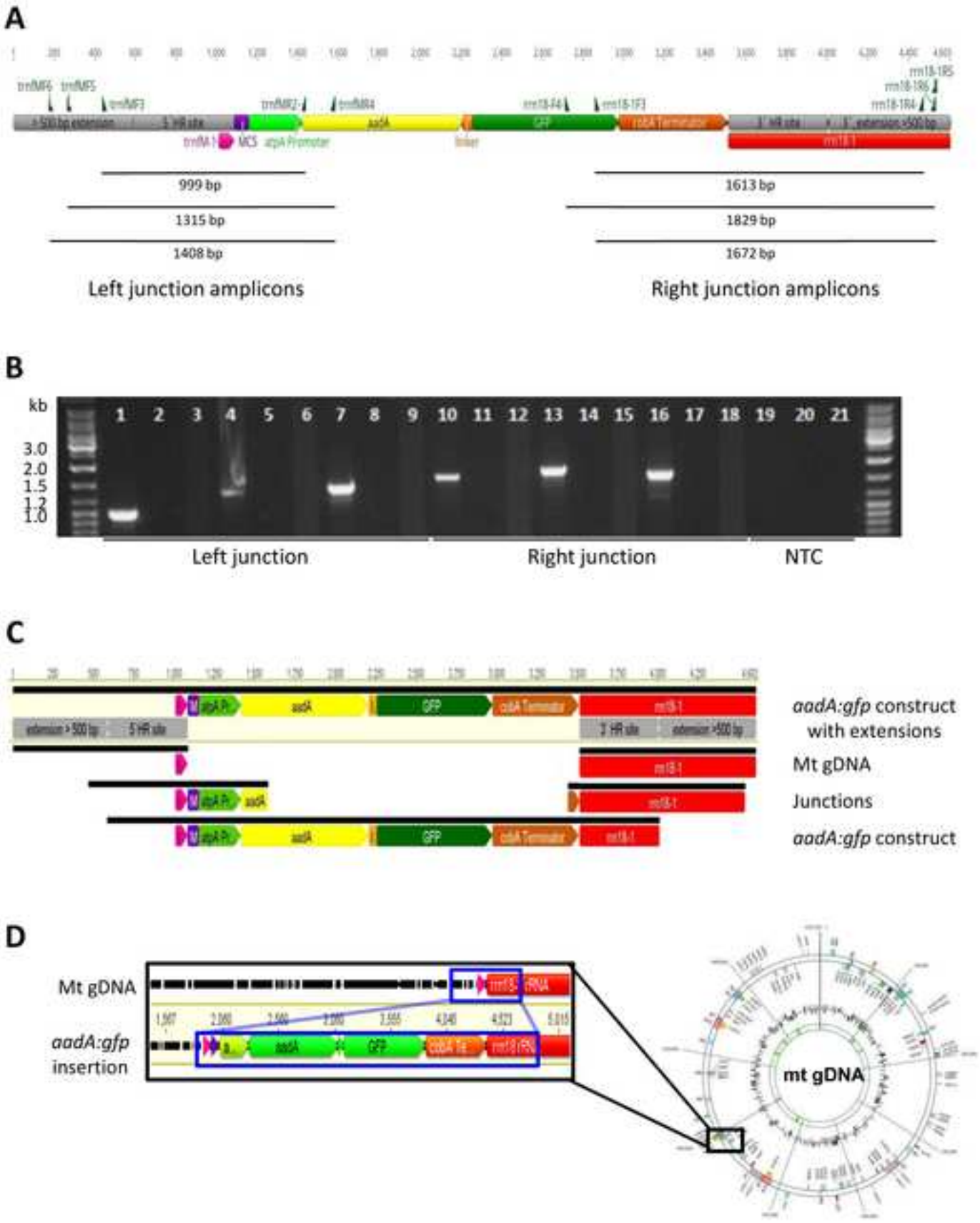

Figure 6

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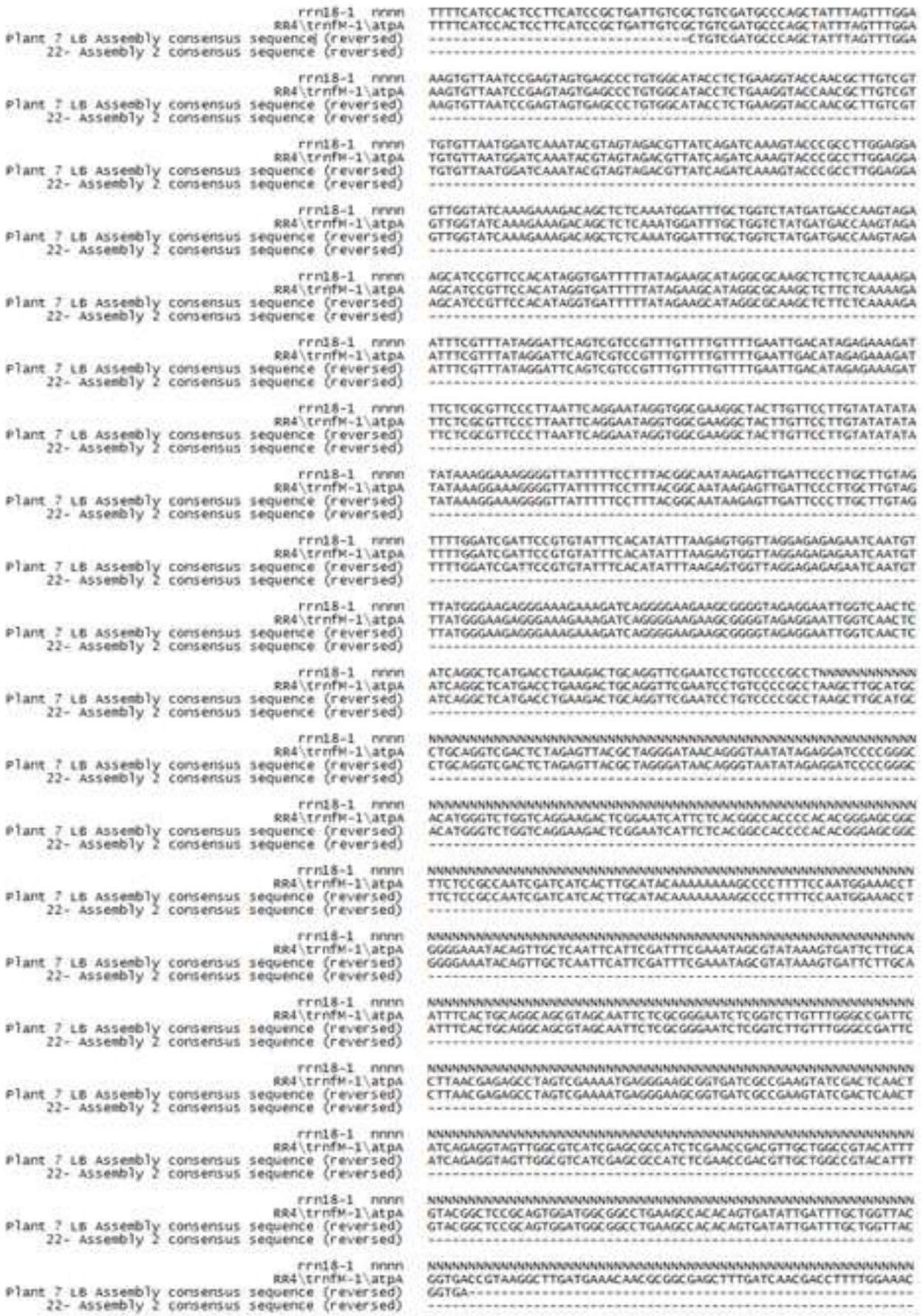

Figure 7

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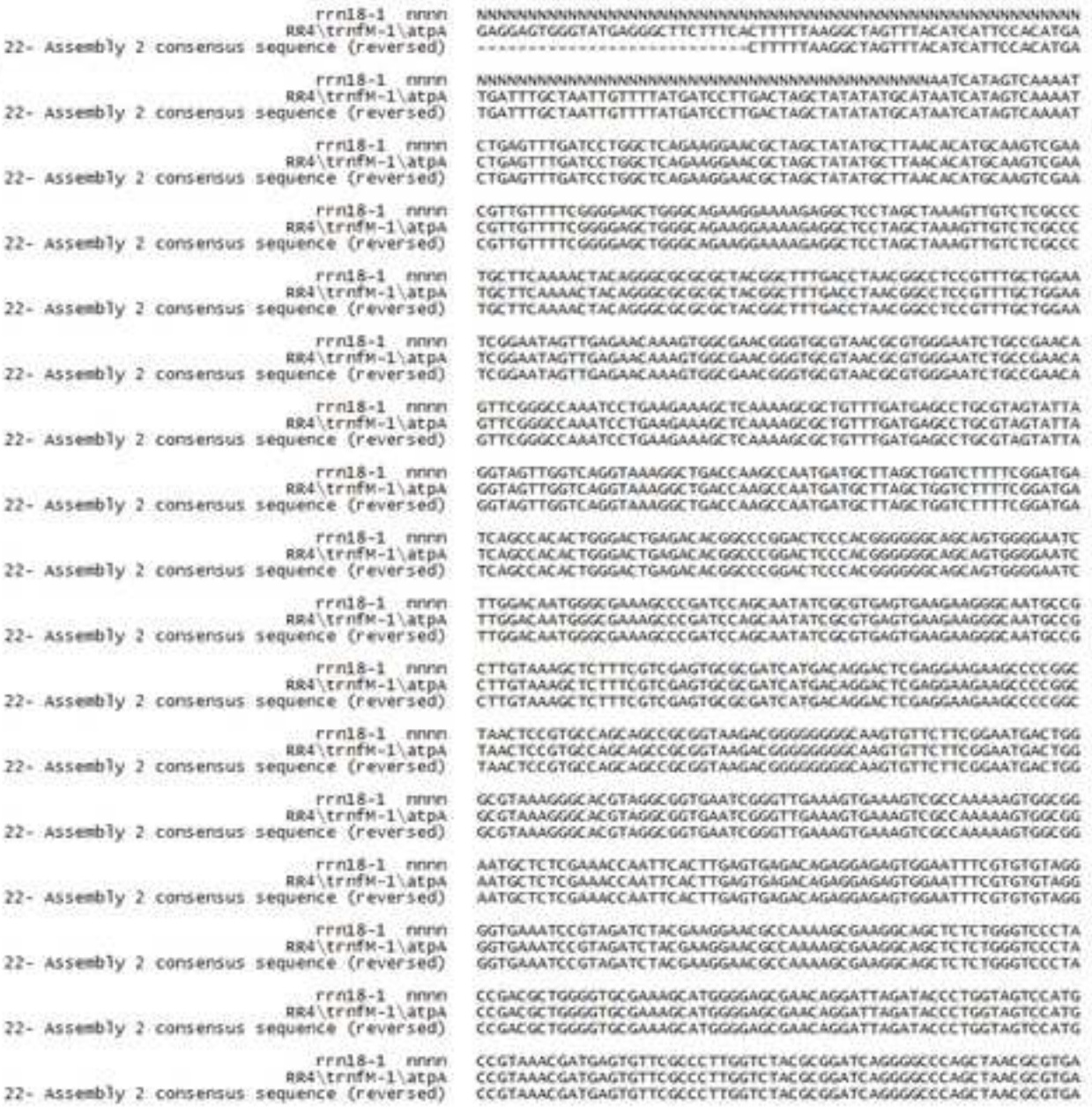

Table 1. Preparation of the peptide working solution for a si

Peptide	Peptide stock 1 mg/mL (µL)	Water (µL)
mTP1	21.6	28.4
mTP2	21.6	28.4
mTP3	34.8	15.2
mTP4	30.0	20.0
mTP5	24.6	25.4

ingle transfection.

Total volume (μL)
50
50
50
50
50

Table 2. PCR program and sample composition fo

RT-PCR program
Step
Initial denaturation
40 Cycles
Dissociation curve
Hold
Reaction composition
Component
2x RT-PCR Master Mix including polymerase (see Table of Materials)
10 μM Forward primer
10 μM Reverse primer
Template cDNA
Nuclease-free water

r the real-time RT-PCR analysis.

Temperature	Time
95°C	15 min
94°C	15 s
58°C	30 s
72°C	30 s
1.9°C/s to 95°C	15 s
1.6°C/s to 60°C	1 min
0.05°C/s to 95°C	15 s
4°C	

20 μL reaction	Final concentration
10 μL	1x
1 μL	0.1 μM
1 μL	0.1 μM
3 μL	variable
5 μL	not applicable

Table 3 Primer sequences

Primer name	Sequence
GFP1L	cacggcagacaaacaaaaga
GFP1R	aaagggcagattgtgtggac
EF F1	gatgacaccaacagccacag
EF R1	ggtgatgctggcatagtgaa
trnfMF3	tttcatccactccttcatcc
trnfMF5	cccttcaattggtcatgctt
trnfMF6	gctgccctacttcatccaag
trnfMR2	gatcaccgcttccctcat
trnfMR4	tcaccgtaaccagcaaatca
rrn18-1F3	gtccacacaatctgcccttt
rrn18-1F4	tcacggcagacaaacaaaag
rrn18-1R4	cctttgagtttcggtcttgc
rrn18-1R5	aaggttttgcgcgttgtatc
rrn18-1R6	ttgcgcgttgtatcgaatta

Binding site
gfp
gfp
EF1a
EF1a
upstream *trnfM-1*
upstream *trnfM-1*
upstream *trnfM-1*
aadA
aadA
gfp
gfp
rrn18-1
rrn18-1
rrn18-1

Table 4. PCR program and sample composition fo

PCR program
Step
Initial denaturation
30 Cycles
Final extension
Hold
Reaction composition
Component
10x CL PCR buffer (Table of Materials)
10 mM dNTPs
10 μM Forward primer
10 μM Reverse primer
Template gDNA (40-80 ng)
Taq polymerase (5 units/μL)
Nuclease-free water

r the end-point PCR analysis.

Temperature	Time
94°C	3 min
94°C	1 min
60°C	30 s
72°C	30 s
72°C	10 min
4°C	

20 μL reaction	Final concentration
2 μL	1x
1 μL	0.5 mM
2 μL	0.1 μM
2 μL	0.1 μM
2 μL	variable
0.125 μL	0.625 units
10.875 μL	not applicable

Table 5 Sequence

mTP
mTP1
mTP2
mTP3
mTP4
mTP5

e and properties of mTPs.

Peptide sequence
MFSYLPRYPLRAASARALVRATRPSYRSALLRYQ
MAAWMRSLFSPLKKLWIRMH
MKLLWRLILSRKW
MWWRRSRTNSLRYT
MLFRLRRSVRLRGLLA

DNA Binding Ratio (w:w)
3.6
3.6
5.8
5.0
4.1

Nuclease Protection Ratio (w:w)
16.0
15.6
25.0
22.4
8.4

Table of Materials

Click here to access/download;Table of Materials;MacMillan et al_Table_of_Materials_R1.xlsx

Name of Material/Equipment	Company	Catalog Number
Chemicals		
$(NH4)_2SO_4$	Sigma	A3920
2,4-Dichlorophenoxyacetic acid (2,4-D)	Sigma	D8407
Agar	Sigma	A7921
a-ketoglutaric acid	Sigma	K1750
Arabinogalactan (Larcoll)	Sigma	L0650
Ascorbic acid	Sigma	A4544
Betaine chloride	Sigma	B7045
Biotin	Sigma	B3399
$CaCl_2$, $2H_2O$	Sigma	C7902
Choline-HCL	Sigma	C-1879
Citric acid	Sigma	C4540
CoCl, $6H_2O$	Fisher	C371
$CuSO_4$, $5H_2O$	Fisher	C489
FeSO4, 7H2O	Sigma	F8263
Ficoll	Sigma	F4375
Folic Acid	Sigma	F8509
Fumaric acid	Sigma	F8509
Gelrite	Sigma	G1910
Glutamine	Sigma	G8540
H_3BO_3	Sigma	B9545
KH_2PO_4	Sigma	P5655
KH_2PO_4	Sigma	P9791
KI	Sigma	P8166
Kinetine	Sigma	K0753
KNO_3	Sigma	P8291
Malic acid	Sigma	M1000
Maltose	Sigma	M5895
$MgSO_4$, $7H_2O$	Sigma	M5921

MitoTracker Orange CM-H_2TMRos	Invitrogen	M7511
$MnSO_4$, H_2O	Sigma	M7899
Myo-Inositol	Sigma	I3011
Na_2EDTA	Sigma	E5134
NH_4NO_3	Sigma	A3795
Nicotinic acid	Sigma	N0761
Pantothenate	Sigma	A7219
Phenylacetic acid (PAA)	Sigma	P6061
Plant preservative mixture (PPM)	Plant Cell Technologies	
Pyridoxine- HCl	Sigma	P8666
Pyruvic acid	Sigma	P2256
Riboflavin	Sigma	C4540
Ribose	Sigma	R7500
Spermidine	Sigma	5292
Spermine	Sigma	S4264
Streptomysin sulphate	Sigma	S9137
Succinic acid	Sigma	S7501
Sucrose	Sigma	S7903
Thiamine-HCl	Sigma	T1270
U2.5 Amino Acid Mixture	sigma	U-7756
Xylose	Sigma	X1500
$ZnSO_4$, $7H_2O$	Sigma	Z1001
Kits & Enzymes		
All Prep DNA/RNA mini kit	Qiagen	80204
DNeasy Plant Mini Kit	Qiagen	69106
GeneRuler 1 kb DNA Lader	Thermo Scientific	11823963
GeneRuler Low Range DNA Lader	Thermo Scientific	10212840
pCR 2.1 Vector TA Cloning Kit	Invitrogen	K204001
Plasmid Maxi kit	Qiagen	12163
QIAquick Gel extraction kit	Qiagen	28706
QIAquick PCR purification kit	Qiagen	28104
QuantiTect SYBR Green PCR Master Mix	Qiagen	204143

RNase-free DNase set	Qiagen	79256
SuperScript III First-Strand Synthesis Kit	Invitrogen	18080051
Taq DNA polymerase kit	Qiagen	201205
Other materials		
150 mm Petri dishes	ThermoFisher	150350
35 mm Petri dishes	Fisher Scientific	0875100A
60 mm Petri dishes	Fisher Scientific	0875113A
90 mm Petri dishes	ThermoFisher	101IRR20
Cell Lyzer	Bertin Technologies	
Magenta vessels	Sigma	V 8505
Semi-transparent flexible film (Parafilm)	ThermoFisher	5833-0001
Sylvania Gro-lux wide spectrum bulbs (40 watts)	Sylvana	
Vacucap 60PF 0.2 UM	VWR	CA28139-704

Comments/Description
Component of 10x Macrosalts solution
Component of NPB-99 medium
Solidifying agent 2; Component of rooting medium
Component of GEM medium
Component of NPB-99 and GEM media
Component of 100x GEM vitamins solution
Component of 100x GEM vitamins solution
Component of 100x GEM vitamins solution
Component of 10x Macrosalts solution
Component of 100x GEM vitamins solution
Component of GEM medium
Component of 100x Microsalts solution
Component of 100x Microsalts solution
Component of 100x Fe-EDTA solution
Component of NPB-99 culture medium
Component of 100x GEM vitamins solution
Component of GEM medium
Solidifying agent 1; Component of GEM medium
Component of NPB-99 and GEM media
Component of 100x Microsalts solution
Component of 10x Macrosalts solution
Component of 10x Macrosalts solution
Component of 100x Microsalts solution
Component of NPB-99 medium
Component of 10x Macrosalts solution
Component of GEM medium
Component of NPB-99 and GEM media
Component of 10x Macrosalts solution

Mitochondria-specific dye for confocal microscopy analysis
Component of 100x Microsalts solution
Component of NPB-99 and GEM media
Component of 100x Fe-EDTA solution
Component of rooting medium
Component of 100x NPB-99 and GEM vitamins solutions
Component of 100x GEM vitamins solution
Component of NPB-99 and GEM media
Component of GEM medium
Component of 100x NPB-99 and GEM vitamins solutions
Component of GEM medium
Component of 100x GEM vitamins solution
Component of GEM medium
Component of GEM medium
Component of GEM medium
Selection agent
Component of GEM medium
Component of GEM medium and rooting medium
Component of 100x NPB-99 and GEM vitamins solutions
Component of GEM medium
Component of GEM medium
Component of 100x Microsalts solution

gDNA and RNA extraction
gDNA isolation
DNA size marker for agarose elecrtophoresis
DNA size marker for agarose elecrtophoresis
Cloning of PCR amplicons
Plasmid isolation
Extraction of PCR products from agarose gels
Purification of linearized plazmid
Real time RT-PCR

Dnase I treatment
cDNA synthesis
Standard PCR

Microspore culture, multi 35 mm Petri dish container
Microspore culture
Microspore culture, water reservoir
Embryo germination
Disruption and homogenization of plant cells and tissues
Plantlet rooting
Plate sealing
Growth chamber lights
Filter sterilization of media and solutions

Manuscript JoVE #60580R1 – Authors' response to the editorial comments

Editorial comments:
The manuscript has been modified and the updated manuscript, **60580_R1.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

The updated version of the manuscript was used for revisions.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been proofread and a few punctuation, grammar and spelling errors have been corrected.

2. Please avoid long steps (more than 4 lines).

Steps longer than 4 lines have been divided into shorter sub-steps.

3. Please do not highlight a step without highlighting any of the sub-steps (step 2.1).

We previously highlighted step 2.1 without highlighting its sub-steps, because step 2.1 contains information important for the method success, while sub-steps contain technical details. We followed the Editor' suggestions (# 3 and 4) and un-highlighted step 2.1. However, we hope that information about the reporter cassette design for mitochondrial transformation will be included in the video script, e.g. in the introductory section.

4. The highlighted protocol steps are over the 2.75 page limit (including headings and spacing). Please highlight fewer steps for filming.

Fewer steps have been highlighted in the revised manuscript for filming by eliminating step 2.1 and replacing step 8 with step 7.1. Compilation of all highlighted steps generated text that is shorter than 2.75 pages (see the attached file 60580_R1_JoVE_Highlighted text for video). However, this change will allow us to show only one representative result (Figure 4), along with a scheme of the protocol (Figure 1).

2. Preparation of DNA and peptides

2.2. DNA cargo preparation

2.1.4. Per each transfection, aliquot 10 µL of the 150 ng/µL DNA stock solution (total DNA amount: 1.5 µg) into sterile 1.5 mL tube.

2.1.5. Add 40 µL of nuclease-free/protease-free H_2O into each tube (final volume: 50 µL) and mix the tube content by gentle pipetting.

Note: Scale up for multiple transfections by preparing the DNA working stock solution in a volume proportional to the number of transfections.

2.2. mTP peptide carrier preparation

2.2.4. Prepare the working stock solution by aliquoting the nuclease-free/protease-free H_2O into sterile 1.5 mL tubes, adding the appropriate volume of the 1 mg/mL peptide stock (**Table 1**), and mix gently.

Note: Scale up for multiple transfections by preparing the peptide working stock solution in a volume proportional to the number of transfections.

3. Transfection of triticale microspores with mTP-DNA nano-complexes (Figure 1B)

3.1. Formation of mTP-DNA nano-complexes

3.1.1. Aliquot 50 µL of the DNA working stock solution into a sterile 1.5 mL tubes and add 50 µL of the peptide working stock solution, mix gently.

3.1.2. Incubate the peptide-DNA mixture for 10 min at room temperature; mix gently by tapping the tube bottom every 5 min.

Note: Do not exceed 15 min, because prolonged incubation will result in aggregation of the peptide-DNA complexes.

3.2. Delivery of mTP-DNA nano-complexes into triticale microspores

3.2.1. Aliquot 500 µL of the 2 x 10^5 cells/mL microspore cell suspension (**Protocol 1.2**; total 100,000 cells per transfection) into sterile 2 mL tubes.

3.2.2. Add 100 µL of the peptide-DNA mixture (**Protocol 2.1**) to microspores and mix gently.
Note: Include control treatments by omitting the peptide or DNA component, or both.

3.2.3. Incubate the peptide-DNA-microspores transfection mixture for 1 h at room temperature; mix gently by tapping the tube bottom every 5 min.

3.2.4. Add 400 µL of the NPB-99 medium (**Protocol 1.1.1**) and continue incubation at room temperature for 24 h (confocal microscopy) or 48 h (qRT-PCR) or 1 h (microspore culture and selection).

Note: Samples for confocal microscopy analysis should be incubated in the dark.

6. Selection and regeneration of putative transformants

6.1. Microspore embryogenesis

6.1.1. Incubate the transfected and control microspores for 1h at room temperature and then transfer to 35 mm Petri dishes containing 2.5 mL NBP-99 medium (total volume: 3.5 mL) and 4 ovaries. Add streptomycin (7 µL of 50 mg/mL stock; final concentration: 100 mg/L).

6.1.2. Seal 35 mm plates with semi-transparent flexible film and place them in a 150 mm Petri dish with 60 mm dish containing distilled water. Seal 150 mm dish with semi-transparent flexible film to keep moisture in.

6.1.3. Culture cells in the dark at 27 °C to induce embryogenesis.

Note: Carry microspore control cultures with or without streptomycin to determine the effect of antibiotics on microspore embryogenesis and green plant regeneration.

6.2. Embryo germination

6.2.1. After four weeks of culture, transfer the developing embryos onto GEM plates supplemented with streptomycin (final concentration: 200 mg/L).

6.2.2. Culture embryos at 16 °C beneath wide spectrum 40 watts bulbs delivering 80 µM m^{-2} s^{-1} (a 16-h light period) for embryo germination.

Note: Only a few embryos will germinate into green plantlets. Some embryos will develop into albino plants or may form roots only, while the majority of embryos will be aborted.

6.3. Rooting of green plantlets

6.3.1. After 3 to 4 weeks, transfer green plantlets into magenta vessels containing the rooting medium with streptomycin (final concentration: 400 mg/L) and continue plantlet cultivation as described above (**6.2.2**).

Note: This selection procedure results in regeneration of haploid putative *aadA:gfp* plants. In order to obtain fertile, doubled haploid plants, haploid plants may be treated with colchicine to induce chromosome duplication[38,39] or cultivated in soil for spontaneous genome duplication.

7. PCR screening of putative transformants

7.1. gDNA isolation

7.1.1. Collect about 100 mg leaf samples from the regenerated plants, freeze the tissue samples in liquid nitrogen and store at -80 °C until use.

7.1.2. Disrupt frozen cells with ceramic or metal beads by shaking aggressively in a cell/tissue disrupting machine (2 times 2 pulses for 60 s at 4,000 x *g*) or by crushing leaf tissue with a pestle in tubes frozen in liquid nitrogen.

7.1.3. Isolate genomic DNA from young leaves of the primary transformants (T_0) at the end of *in vitro* culture steps using a kit summarized in the **Table of Materials**, following the manufacturer's protocol.

7.2. PCR

7.2.1. Perform PCR using GFP1L/GFP1R primers (**Table 3**) according to the protocol outlined in **Table 4**.

7.2.2. Separate the PCR samples by electrophoresis in a 1.0% agarose gel containing ethidium bromide in 1 × TAE buffer for 1 h.

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"

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