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Transforming, Genome Editing and Phenotyping the Nitrogen-Fixing Tropical Cannabaceae Tree Parasponia andersonii

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TITLE:

Transforming, Genome Editing and Phenotyping the Nitrogen-Fixing Tropical Cannabaceae Tree *Parasponia andersonii*

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KEYWORDS:

Parasponia andersonii, Cannabaceae, tree, wood, *Agrobacterium tumefaciens*, stable transformation, CRISPR-Cas9, genome editing, nodulation, rhizobium, mycorrhization

SUMMARY:

Parasponia andersonii is a fast-growing tropical tree that belongs to the Cannabis family (Cannabaceae) and can form nitrogen-fixing root nodules in association with the rhizobium. Here, we describe a detailed protocol for reverse genetic analyses in *P. andersonii* based on *Agrobacterium tumefaciens*-mediated stable transformation and CRISPR/Cas9-based genome editing.

ABSTRACT:

Parasponia andersonii is a fast-growing tropical tree that belongs to the Cannabis family (Cannabaceae). Together with 4 additional species, it forms the only known non-legume lineage able to establish a nitrogen-fixing nodule symbiosis with rhizobium. Comparative studies

between legumes and *P. andersonii* could provide valuable insight into the genetic networks underlying root nodule formation. To facilitate comparative studies, we recently sequenced the *P. andersonii* genome and established *Agrobacterium tumefaciens*-mediated stable transformation and CRISPR/Cas9-based genome editing. Here, we provide a detailed description of the transformation and genome editing procedures developed for *P. andersonii*. In addition, we describe procedures for the seed germination and characterization of symbiotic phenotypes. Using this protocol, stable transgenic mutant lines can be generated in a period of 2-3 months. Vegetative in vitro propagation of T_0 transgenic lines allows phenotyping experiments to be initiated at 4 months after *A. tumefaciens* co-cultivation. Therefore, this protocol takes only marginally longer than the transient *Agrobacterium rhizogenes*-based root transformation method available for *P. andersonii*, though offers several clear advantages. Together, the procedures described here permit *P. andersonii* to be used as a research model for studies aimed at understanding symbiotic associations as well as potentially other aspects of the biology of this tropical tree.

INTRODUCTION:

Parasponia andersonii is a tropical tree belonging to the Cannabis family (Cannabaceae) and is native to Papua New Guinea and several Pacific Islands[1–3]. Together with 4 additional *Parasponia* species, it represents the only non-legume lineage that can establish a nitrogen-fixing nodule symbiosis with rhizobia. This symbiosis is well studied in the legume (Fabaceae) models *Medicago truncatula* and *Lotus japonicus*, which has resulted in acquiring detailed knowledge of the molecular genetic nature of nodule formation and functioning[4]. Additionally, it was demonstrated that the root nodule symbiosis in legumes is founded on the much older, and widespread arbuscular mycorrhizal symbiosis[5]. Phylogenomic comparisons suggest that the nitrogen-fixing nodule symbioses of legumes, *Parasponia*, as well as, the so-called actinorhizal plant species that host diazotrophic *Frankia* bacteria, have a shared evolutionary origin[6–8]. To determine whether the genes identified to be involved in the legume nodule formation are the part of a conserved genetic basis, studies on non-legume species are essential. To this end, we propose to use *P. andersonii* as a comparative research model, alongside legumes, to identify the core genetic networks underlying root nodule formation and functioning.

P. andersonii is a pioneer that can be found on the slopes of volcanic hills. It can meet growth speeds of 45 cm per month and reach lengths of up to 10 meters[9]. *P. andersonii* trees are wind-pollinated, which is facilitated by the formation of separate male and female flowers[3,10]. We recently sequenced and annotated the diploid genome (2n = 20; 560 Mb/1C) of *P. andersonii*, and assembled draft genome sequences of 2 additional *Parasponia* species; *P. rigida* and *P. rugosa*[6]. This revealed ~35,000 *P. andersonii* gene models that can be clustered in >20,000 orthogroups together with genes from *M. truncatula*, soybean (*Glycine max*), *Arabidopsis thaliana*, woodland strawberry (*Fragaria vesca*), *Trema orientalis,* black cotton poplar (*Populus trichocarpa*) and eucalypt (*Eucalyptus grandis*)[6]. Additionally, transcriptome comparisons between *M. truncatula* and *P. andersonii* identified a set of 290 putative orthologues that display a nodule-enhanced expression pattern in both species[6]. This provides an excellent resource for comparative studies.

To study the gene function in *P. andersonii* roots and nodules, a protocol for *Agrobacterium rhizogenes*-mediated root transformation has been established[11]. Using this protocol, compound plants bearing transgenic roots can be generated in a relatively short time frame. This method is, also, widely applied in the legume-symbiosis research[12–14]. However, the disadvantage of this method is that only roots are transformed and that each transgenic root represents an independent transformation event, resulting in substantial variation. Also, the transformation is transient and transgenic lines cannot be maintained. This makes *A. rhizogenes*-based root transformation less suited for CRISPR/Cas9-mediated genome editing. Additionally, *A. rhizogenes* transfers its *root inducing locus* (*rol*) genes to the plant genome, which once expressed interfere with hormone homeostasis[15]. This makes studying the role of plant hormones in *A. rhizogenes*-transformed roots challenging. To overcome these limitations, we recently developed a protocol for *Agrobacterium tumefaciens*-based transformation and CRISPR/Cas9-mediated mutagenesis of *P. andersonii*[10].

Here, we provide a detailed description of the *A. tumefaciens*-based transformation procedure and reverse genetics pipeline developed for *P. andersonii*. Additionally, we provide protocols for the downstream handling of transgenic plantlets, including assays to study symbiotic interactions. Using the protocol described here, multiple transgenic lines can be generated in a 2-3 months period. In combination with CRISPR/Cas9-mediated mutagenesis, this allows efficient generation of knockout mutant lines. These mutant lines can be vegetatively propagated in vitro[10,16,17], which allows sufficient material to be generated to start phenotypic characterization at 4 months after the transformation procedure has been initiated[10]. Together, this set of procedures should allow any lab to adopt *P. andersonii* as a research model for studies aimed at understanding rhizobial and mycorrhizal associations, as well as potentially other aspects of the biology of this tropical tree.

PROTOCOL:

1. Grow *P. andersonii* trees in the greenhouse

1.1. Germinate *P. andersonii* WU1 seeds[18].

1.1.1. Use fresh *Parasponia* berries or soak dried berries in water for 2 h to rehydrate. Squash berries on a piece of tissue paper or rub against the inside of a tea sieve to remove the seeds.

1.1.3. Disinfect seeds using commercial bleach (~4% hypochlorite) for 15-20 min and subsequently wash the seeds 6 times using sterilized water.

1.1.4. Transfer the seeds to sterile 200 µL PCR tubes. Fill the tubes with sterilized water, such that the seeds are completely submerged. Incubate the tubes for 10 days in a thermocycler running the following program: 30 cycles (7 °C for 4 h, 28 °C for 4 h). Do not use a heated lid, as this might kill the seeds.

1.1.5. Prepare SH-0 plates (see **Table 1**). Transfer the seeds to SH-0 plates and incubate at 28 °C,

16 h:8 h day:night. Close plates with 2 layers of elastic sealing foil to prevent drying during incubation at 28 °C.

1.2. After seedlings have developed their first set of true leaves (~3-4 weeks after incubation at 28 °C), transfer seedlings to pots filled with commercial potting soil and cover the seedlings with a translucent plastic cup to prevent desiccation. Place pots in a 28 °C climate room or greenhouse, ~85% RH, under a 16 h:8 h day:night regime.

1.3 After 1 week, remove the translucent plastic cup. Water the pots regularly and when trees grow bigger supplement with fertilizer to sustain growth.

2. Cloning of constructs for CRISPR/Cas9-mediated mutagenesis of *P. andersonii*

NOTE: Standard binary transformation vectors can be used for the stable transformation of *P. andersonii*. Here, as an example, is a procedure to generate constructs for CRISPR/Cas9-mediated mutagenesis using modular cloning (e.g., Golden Gate)[19].

2.1. Identify guide RNA target sequences for the gene(s) of interest, using bioinformatics software featuring a built-in CRISPR design tool. Choose guide RNA sequences located at the 5'-end of the coding sequence of the target gene to increase the chance of obtaining full knockouts. Make sure to check for off-target effects by searching against the *P. andersonii* genome[6].

NOTE: Use 2 sgRNAs per target gene, preferably 200-300 bp apart. This may generate deletions that can be identified by PCR and subsequently by agarose gel electrophoresis.

2.2. Generate level 1 Golden Gate constructs containing the sgRNA sequences.

2.2.1. Design primers to amplify each individual sgRNA by inserting the 20 bp guide sequence at the position of $\mathbf{N_{(20)}}$ in the following primer sequence: 5'-TGTGGTCTCAATTG$\mathbf{N_{(20)}}$ GTTTTAGAGCTAGAAATAGCAAG-3'.

NOTE: If the guide sequence equals $GN_{(19)}$, remove the G at the 5' end of the guide sequence before inserting in the primer sequence.

2.2.2. PCR amplify sgRNAs from pICH86966::AtU6p::sgRNA_PDS[20] using the forward primers designed at step 2.2.1 and the universal reverse primer: 5'-TGTGGTCTCAAGCGTAATGCCAACTTTGTAC-3'. Use a high-fidelity heat-stable DNA polymerase and the following PCR conditions: 98 °C for 30 s; 30 cycles (98 °C for 10 s; 53 °C for 20 s; 72 °C for 10 s); 72 °C for 7 min. Successful PCR reactions yield a 165 bp amplicon.

2.2.3. Column-purify the PCR amplicon using a commercial PCR purification kit. Subsequently, set up Golden Gate reactions to clone sgRNAs behind the *Arabidopsis thaliana* AtU6p small RNA promoter: 10 ng of the sgRNA PCR amplicon, 150 ng of pICSL01009::AtU6p[20], 60 ng of the appropriate level 1 acceptor vector, 2 µL of T4 ligase buffer, 2 µL 0.1% of bovine serum albumin

(BSA), 0.5 µL of BsaI, 0.5 µL of T4 ligase, fill to 20 µL with ultra-pure water. Ensure that all sgRNAs are cloned in the same orientation to prevent hairpin formation.

2.2.4 Incubate reactions in a thermocycler running the following program: 37 °C for 20 s; 26 cycles (37 °C for 3 min; 16 °C for 4 min); 50 °C for 5 min; 80 °C for 5 min. Transform Golden Gate reactions to *Escherichia coli* and plate on LB medium[21] containing ampicillin (50 mg/L), X-Gal (200 mg/L) and IPTG (1 mM).

NOTE: Prepare stock solutions of IPTG and X-Gal in ultra-pure water and dimethylformamide, respectively. Filter sterilize the ampicillin and IPTG stock solutions and store all stocks at -20 °C. Wear gloves when handling dimethylformamide.

2.2.5. Select white colonies and isolate plasmids using a commercial plasmid isolation kit. Sequence verify isolated plasmids before continuing with Golden Gate level 2 assembly.

2.3. Assemble level 2 Golden Gate constructs for the stable transformation.

2.3.1. Perform a Golden Gate reaction using the level 1 AtU6p::sgRNA constructs (generated under section 2.2) as well as pICH47802::*NPTII*, pICH47742::35S$_{pro}$::ΩNLS-aCas9::35S$_{ter}$, the level 2 acceptor pICSL4723 and the appropriate end-linker (see Engler et al.[22]). Perform reactions as following: use ~100 fmol of each donor vector and ~20 fmol of the acceptor vector and add 2 µL of T4 ligase buffer, 2 µL of 0.1% BSA, 0.5 µL of BpiI, 0.5 µL of T4 ligase, fill to 20 µL with ultra-pure water.

NOTE: The level 1 plasmids pICH47802::*NPTII*, pICH47742::35S$_{pro}$::ΩNLS-aCas9::35S$_{ter}$ need to be cloned first (see **Supplemental File 1**), as is described for sgRNAs under section 2.2[20,22,23].

2.3.2. Incubate reactions as under step 2.2.4 and transform into *E. coli*. Plate on LB medium containing kanamycin. Next day, select white colonies and isolate plasmids. Determine the correct plasmid assembly by restriction-digestion analysis.

2.4. Transform level 2 constructs to *Agrobacterium tumefaciens* strain AGL1[24].

3. Stable transformation of *P. andersonii*

3.1. Inoculate 2 LB plates containing the appropriate antibiotics with *A. tumefaciens* strain AGL1 transformed with the construct of interest. Incubate plates at 28 °C for 2 days.

3.2. Harvest young branches from greenhouse-grown trees. Use about 5 branches of 5-8 cm in length for each transformation. Ensure to only use healthy non-infected branches. Remove the leaves by cutting them as such that ~1 cm^2 of leaf tissue is left at the end of each petiole. Discard the leaves.

3.3. Disinfect tissue for 15 min using 1:1-diluted commercial bleach (~2% hypochlorite after

dilution) containing a few drops of polysorbate 20. Then, rinse the tissue 6 times with autoclaved water.

NOTE: This step, as well as, the following steps need to be conducted inside a laminar down-flow cabinet to keep tissue sterile.

3.4. Re-suspend the *A. tumefaciens* cells from 1-2 plates in 25 mL of infiltration medium (see **Table 1**) containing acetosyringone (20 mg/L) and a non-ionic surfactant (0.001% v/v) to reach an optical density (OD_{600}) of ~5.

NOTE: Prepare the acetosyringone stock solution in 70% ethanol and store at -20 °C. The non-ionic surfactant needs to be filter-sterilized before adding to the infiltration medium.

3.5. Cut both the stem and petiole tissue in pieces of ~1 cm in length inside the *A. tumefaciens* suspension, thereby creating fresh wounds at both sides. Leave tissue pieces in the *A. tumefaciens* suspension for 10-30 min.

3.6. Prepare the rooting medium (see **Table 1**) and add acetosyringone (20 mg/L) after autoclaving. Dry tissue pieces on a sterile piece of filter paper and place it on the medium (~10 explants/plate). Incubate plates in dark at 21 °C for 2 days.

NOTE: Allow the medium to cool down to ~60 °C prior to adding acetosyringone.

3.7. After 2 days, inspect plates for fungal or obvious bacterial contamination (bacteria other than *A. tumefaciens*). Contaminated plates need to be discarded.

3.8. Prepare liquid SH-10 medium (see **Table 1**). After autoclaving, add polysorbate 20 (0.01%, v/v). Transfer tissue pieces to 10 mL of SH-10 containing polysorbate 20. During a period of at least 10 min, gently agitate every 2-3 min to wash the tissue.

3.9. Wash two additional times with fresh SH-10 containing polysorbate 20. These times, a 2-3 min incubation time per washing step is enough.

3.10. Prepare the rooting medium (see **Table 1**). After autoclaving, add cefotaxime (300 mg/L) and kanamycin (50 mg/L) and pour plates. For the secondary transformations (transformations of transgenic kanamycin-resistant lines), apply hygromycin (15 mg/L) selection.

3.11. Dry tissue pieces on sterile pieces of filter paper. Afterwards, transfer tissue pieces to the plates prepared in step 3.9.

3.12. Incubate plates for 7 days at 28 °C, 16 h:8 h day:night. Every 2 days check plates for fungal or bacterial contamination and excessive growth of *A. tumefaciens*. In case of contamination, transfer non-infected pieces to a fresh plate.

3.13. After 7 days, transfer tissue pieces to propagation medium (see **Table 1**) containing cefotaxime (300 mg/L) and kanamycin (50 mg/L). Incubate plates at 28 °C, 16 h:8 h day:night. Refresh plates once a week until transgenic shoots develop. Ensure to only transfer non-infected tissue pieces to fresh plates. Discard the pieces that are overgrown by *A. tumefaciens*.

3.14. Once putatively-transgenic shoots are ≥1 cm in length, cut shoots and culture them independently in the propagation medium containing cefotaxime (300 mg/L) and kanamycin (50 mg/L). To ensure that shoots represent independent transformants, take only a single shoot from each side of an explant.

3.15. Vegetatively propagate putatively-transgenic shoots as described under step 5.2.

4. Genotyping of putatively-transgenic shoots

4.1. Design primers spanning the sgRNA recognition site(s). To allow PCR amplicon sequencing, choose primers 150-250 bp away from the sgRNA recognition site(s).

4.2. Cut a leaf tip (~5 mm) from each transgenic shoot to be genotyped. Also, harvest a wild-type control sample.

4.3. Perform 50 µL PCR reactions using the primers designed at step 4.1 and a commercial kit to directly amplify DNA from plant samples. Alternatively, PCR reactions can be performed on purified DNA using a high-fidelity polymerase.

4.4. Separate PCR amplicons on a 1.5-2% agarose gel.

4.5. Analyze the results from gel electrophoresis. Check for samples producing multiple bands (more than 1 allele) and PCR amplicons with sizes different from wild type, which indicates the presence of medium-sized indels.

4.6. Sequence PCR amplicons to identify the exact mutations. For samples producing a single PCR amplicon, PCR products can be sequenced directly. Samples that produce more than 1 band after gel electrophoresis or that appear to be heterozygous after direct sequencing of the PCR amplicon, need to be cloned into a blunt-end cloning vector first. Subsequently, sequence multiple clones for each sample to identify all possible alleles present in the sample.

4.7. Align sequencing results to the gene of interest and inspect the alignment to check for mutations near the sgRNA target site(s). Subsequently, check whether these mutations create frameshifts. Discard lines with > 2 alleles, and lines containing in-frame mutations.

4.8. Select several lines for further analysis.

4.9. Propagate selected lines as described under step 5.2.

4.10. When lines have developed several new shoots, take new samples from ≥3 leaf tips and repeat steps 4.3-4.7. Determine whether the mutations present in each of the samples originating from the same line as well as the original PCR sample are identical. Lines that yield the same mutations in all samples are homogeneously mutated and can be used for further experimentation. Discard lines that do not yield the same results as these lines are chimeric.

5. Preparation of rooted *P. andersonii* plantlets for experimentation

5.1. Initiate a new tissue culture line of *P. andersonii*.

5.1.1. Harvest axillary buds, young adventitious shoots or leaf tissue from healthy trees. Alternatively, seedlings can be used as a starting material.

5.1.2. Disinfect tissue using 1:1-diluted commercial bleach (~2% hypochlorite after dilution) containing a few drops of polysorbate 20 for 15 min. Afterwards, rinse tissue 6 times using autoclaved water.

NOTE: This step, as well as, the following steps need to be conducted inside a laminar downflow or laminar crossflow cabinet to keep tissue sterile.

5.1.3. Transfer tissue to propagation medium (see **Table 1**). Close plates with 2 layers of elastic sealing foil and incubate plates at 28 °C, 16 h:8 h day:night.

5.1.4. Inspect plates every few days during the first 2 weeks to ensure that tissue is free from fungal or bacterial contamination.

5.2. Propagate tissue by placing ~10 shoots on a fresh plate of propagation medium and close the plate with 2 layers of elastic sealing foil. Incubate plates at 28 °C, 16 h:8 h day:night. Repeat this step every 4 weeks.

5.3. When shoots are >1 cm in length, cut shoots at their base and place them on the rooting medium (see **Table 1**). About 10 shoots can be placed on a single rooting plate. Position shoots upright by inserting the basal tip of the shoot into the medium. Roots appear at 10-14 days after incubation of the plates at 28 °C, 16 h:8 h day:night.

NOTE: Do not root all shoots but keep part for tissue culture propagation (see step 5.2).

6. Nodulation of *P. andersonii* plantlets in pots

6.1. Prepare rhizobium inoculum.

6.1.1. Inoculate 10 mL of liquid YEM medium (see **Table 2**) from a single colony of *Mesorhizobium plurifarium* BOR2[6] and incubate at 28 °C for 2 days.

NOTE: *M. plurifarium* BOR2 is preferred as it efficiently nodulates *P. andersonii*. However, other rhizobium strains can also be used for nodulation of *P. andersonii* (e.g. *Bradyrhizobium elkanii* WUR3[25], *Rhizobium tropici* CIAT899[26,27] or *Bradyrhizobium* sp. Kelud2A4).

6.1.2. Use the 10 mL culture to inoculate a larger volume of liquid YEM medium. The volume of this culture is dependent on the number of pots that need to be inoculated.

6.1.3. Prepare liquid EKM medium (see **Tables 3, Table 4**). Centrifuge the bacterial culture for 10 min at 3,500 *x g* to harvest the cells. Subsequently, re-suspend the bacterial pellet in liquid EKM (use approximately the same volume as the original YEM culture) and determine the optical density (OD_{600}).

6.2. For ~20 pots, prepare 3 L of liquid EKM medium and inoculate with the rhizobial suspension prepared at step 6.1.3. to reach OD_{600} = 0.025.

6.3. Mix 3 L of EKM containing rhizobia with 1,250 g of perlite. Subsequently, add 210 g of this mixture to sterile translucent polypropylene pots. Alternatively, instead of perlite, use sand as a substrate for nodulation assays.

6.4. Plant 1-3 *P. andersonii* plantlets in each pot. Also, prepare several pots containing *P. andersonii* plantlets transformed with the CRISPR-control construct (see **Supplementary Table 1**). Weigh several pots to be able to determine water loss during the experiment. Cover the bottom of each pot to shield the roots from light exposure.

6.5. Incubate pots in a climatized growth room (28 °C, 16 h:8 h day:night) for 4-6 weeks. Once a week, weigh several pots to determine water loss. If water loss exceeds 10 mL, supplement with ultra-pure water to compensate for the loss.

6.6. After 4-6 weeks, clean the roots from perlite and determine nodule numbers using a binocular to examine the nodulation efficiency.

7. Nodulation of *P. andersonii* plantlets on plates

7.1. Prepare cellophane membranes[28].

7.1.1. Cut the cellophane membrane to fit into a square 12 cm x 12 cm Petri dish. Cut the membranes a bit shorter at the top to allow space for the shoots to grow.

7.1.2. To increase the permeability of cellophane membranes, boil the membranes in EDTA solution (1 g/L) for 20 min. Afterwards, rinse at least 6x with demineralized water to remove the EDTA.

NOTE: As the dry membrane tend to wrinkle when in contact with water, submerge the dry membranes one by one into the solution.

7.1.3. Arrange the membranes horizontally in a thin layer of water in a round glass plate. Sterilize the membranes by autoclaving twice.

7.2. Place 1 autoclaved cellophane membrane on a square 12 x 12 cm Petri dish containing agar-solidified EKM medium (see **Table 3, Table 4**). Place two 3-week old rooted *P. andersonii* plantlets (see section 5) or 4-week old seedlings (see section 1.1) on the top of the membrane. Ensure to only pick plantlets or seedlings with roots that have white root tips, indicating that these roots are still growing.

7.3. Gently cover the roots with a second cellophane membrane, creating a sandwich layer. Seal the plate with 3 layers of elastic sealing foil. Wrap the bottom half of the plates with aluminum foil, to cover the roots from light exposure.

7.4. Incubate the plates in a climatized growth room (28 °C, 16 h:8 h day:night) for 3-4 weeks. Mark the position of the root tips to follow the root growth over time.

7.5. If the EKM plates start to dry out due to prolonged incubation, transfer the plants to fresh EKM plates a few days ahead of bacterial inoculation.

7.6. Prepare the bacterial inoculum as described at step 6.1.

7.7. Remove the top cellophane membrane and apply 1 mL of rhizobium culture (OD_{600} = 0.025) to the roots. Subsequently, place a new cellophane membrane on the inoculated roots. Wrap the outside of the plate using aluminum foil to cover the roots from light exposure.

7.8. After 4 weeks, examine nodule numbers using a binocular to determine the nodulation efficiency.

8. Nodulation of *P. andersonii* seedlings in pouches

8.1. Germinate *P. andersonii* seeds as described in section 1.1. After the cotyledons have fully emerged (~12 days on SH-0 plates at 28 °C), transfer the seedlings to pouches.

8.2. To prepare the pouches, tear the folded section of the paper wick and add 7 mL of modified EKM medium (see **Table 3, Table 4**).

8.3. Insert 1 or 2 seedlings by placing the roots in between both sheets of paper that form the paper wick and the front plastic sheet of the pouch.

8.4. Shield the roots from light exposure, by folding aluminum foil around the pouch. Suspend the pouches in a plastic box covered with a translucent lid to maintain high humidity. Place the box in a climatized growth room (28 °C, 16 h:8 h day:night).

8.5. Compensate for water evaporation by adding sterile ultra-pure water, as such that the paper wick remains humid (avoid standing water at the bottom of the pouch). After the first week, this generally requires adding 2-3 ml every 4 days.

8.6. Prepare the bacterial inoculum as described at step 6.1.

8.7. After seedlings have been grown for 10-12 days in pouches, inoculate the root system with 500 µL of rhizobium culture (OD_{600} = 0.025).

8.8. Follow the nodule formation through time. Four weeks post inoculation, nodules can be counted and harvested to determine nodulation efficiency.

9. Nodule cytoarchitecture analysis

9.1. Collect 10-15 nodules in a 2 mL tube containing fixative (5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2). Apply vacuum for ½-1 h and incubate overnight at 4 °C. During this period, the samples sink to the bottom of the tube.

NOTE: The fixative solution can be stored at 4 °C for ~2-4 weeks prior usage. Make sure to wear gloves when working with tissue fixative.

9.2. Wash the nodules 2x with 0.1 M phosphate buffer, pH 7.2. Apply 10 min intervals between each washing step.

9.3. Dehydrate the samples by subsequently incubating in 30%, 50%, 70%, and 100% ethanol. To ensure that all water is removed from the samples, repeat the 100% ethanol step 3x. Apply 10 min intervals between each dehydration step.

9.4. Prepare polymerization mixture I (PM-I) by adding 1 pack of Hardener I to 2.5 mL of PEG400 mixed with 100 mL of HEMA (2-hydroxyethyl methacrylate)-based resin solution. Stir the solution for ~15 min to completely dissolve the Hardener I. Subsequently, store PM-I at -20 °C.

9.5. Remove the ethanol from step 9.3. and infiltrate the samples in the following order: PM-I:100% ethanol (1:3, v/v), PM-I:100% ethanol (1:1, v/v), and PM-I:100% ethanol (3:1, v/v). Incubate the samples in each solution at RT for ½-1 h or until the samples sink to the bottom.

9.6. Incubate samples overnight at 4 °C in 100% PM-I solution.

9.7. Prepare polymerization mixture II by mixing PM-I and Hardener II in a 15:1 (v/v) ratio. Fill the plastic mold with the polymerization solution, orient the samples horizontally at the bottom of the mold, and cover with a piece of elastic sealing foil. Avoid the formation of air bubbles.

NOTE: As the solution starts to polymerize upon exposure to RT, try to orient the samples as quickly as possible in the plastic holder. Polymerization is completed after overnight incubation

at RT, or 1 h at 37 °C.

9.8. Remove the elastic sealing foil cover from step 9.7 and place a holder to the polymerized samples. To mount the holder to the samples, dissolve 10 mL of methyl methacrylate-based resin powder in 5 mL of methyl methacrylate-based resin solution. Quickly add the solution to the hole in the top of the holder.

NOTE: Perform the polymerization step in the fume hood (~30 min at RT).

9.9. Microtome section samples to a thickness of 4-5 µm. Place a microscope slide on a 58 °C hot plate and add a large drop of water to each slide. Place the sections on the top of the water. Once the water has evaporated, the sections will adhere to the slide.

9.10. Stain slides by immersing in 0.05 % (w/v) toluidine blue for 2 min. Subsequently, rinse slides 3x with ultra-pure water. Slides can be observed using a bright-field microscope.

10. Mycorrhization of *P. andersonii* plantlets

10.1. Prepare *Rhizophagus irregularis* spores' inoculum

10.1.1. Prepare a stack of polyester woven filters with the following sizes (top to bottom): 210 µm, 120 µm, and 36 µm mesh size.

10.1.2. Pipette the required amount of a commercial spore suspension onto the stack of polyester filters. Rinse the filters 3x with 100 mL of autoclaved demineralized water. The spores are retained on the surface of the 36 µm filter.

NOTE: Prepare the spore suspension in the laminar crossflow cabinet to prevent contamination.

10.1.3. Disassemble the polyester stack and keep the 36 µm filter only. Repeat the washing step with autoclaved demineralized water for at least 6x.

10.1.4. Place the filter on a Petri dish and re-suspend the spores in autoclaved demineralized water. Use a volume of water equal to the volume of the spore suspension used in step 10.1.2. Transfer the spore suspension to a sterile tube by pipetting.

10.1.5. Place 5 drops of 20 µL of the spore suspension on a glass slide and count the number of spores using a bright-field microscope. Convert spore counts into a ratio of spores/mL and dilute the spore suspension until it reaches 250 spores/mL. Store the spore suspension at 4 °C.

10.2. Perform mycorrhization assay. To this end, add 800 g of autoclaved sand supplemented with 70 mL of ½-Hoagland medium to sterile translucent polypropylene pots (see **Tables 5**-**6**). Mix sand and medium directly in the pot by shaking vigorously.

10.3. Place one *P. andersonii* plantlet in each pot, and pipette 1 mL of the spore suspension directly onto the root of the *P. andersonii* plantlet. Ensure to include several pots containing *P. andersonii* plantlets transformed with a CRISPR-control construct (see **Supplementary Table 1**).

10.4. Incubate pots in a climatized growth room (28 °C, 16 h:8 h day:night) for 6 weeks.

10.5. Take out the plants from the pots and wash the roots with running water to remove as much sand as possible.

10.6. Cut roots in 1 cm long pieces and boil the root pieces in 10% KOH (w/v) for 20 min at 90 °C. Subsequently, place the boiled roots on a cell strainer with a 100 µm mesh size and rinse 3x with 50 mL of water.

10.7. Stain roots with 0.05% (w/v) trypan blue in lactoglycerol (300 mL of lactic acid; 300 mL of glycerol; and 400 mL of demineralized water) for 5 min at 90 °C in a water bath or heating block. Subsequently, transfer roots to 30% glycerol. The root samples can be stored at RT.

10.8. Place 15-25 root fragments on a single microscope slide. Add 30% glycerol and cover with a cover glass and press until root pieces become flat. Observe the root fragments using a bright-field microscope and score the mycorrhizal colonization.

NOTE: A method to score mycorrhization is described according to Trouvelot et al.[29]. This method uses several classes (%F, %M, and %A), which allows rapid estimation of the level of mycorrhizal colonization of each root fragment and abundance of arbuscules.

REPRESENTATIVE RESULTS:

*P. andersonii t*rees can be grown in a conditioned greenhouse at 28 °C and ~85% relative humidity (**Figure 1A**). Under these conditions, trees start flowering at 6-9 months after planting. Female *P. andersonii* flowers produce berries that each contains a single seed. During maturation, the berries change color; first from green to white and subsequently from white to brown (**Figure 1B**). Seeds extracted from the ripened brown berries, germinate well after a 10-day temperature cycle and a 7-day incubation on SH-0 plates (**Figure 1C**). Germinated seeds continue to develop into young seedlings that can be used for experimentation after ~4 weeks (**Figure 1D**).

We have previously shown that petioles and segments of young *P. andersonii* stems can be efficiently transformed using *A. tumefaciens* strain AGL1[10]. At the start of the transformation procedure, the tissue explants are co-cultivated with *A. tumefaciens* for 2 days at 21 °C (**Figure 2A**). Prolonged co-cultivation results in the over-colonization of the tissue explants by *A. tumefaciens* and should, therefore, be prevented (**Figure 2B**). After the co-cultivation period, tissue explants are transferred to selective media, which promotes outgrowth of transformed tissue. Two to three weeks later, small green micro-calli are generally observed along the original wound surface (**Figure 2C**). These calli should continue to grow and develop 1 or more putatively-transformed shoots at 6-8 weeks after the transformation procedure has been initiated (**Figure 2D**). At this stage, transformation efficiencies typically range from ~10-30% for transformations

initiated with tissue explants taken from mature and partly woody branches (**Table 7**). If transformations are initiated with explants taken from the young and rapidly-growing tips of branches that are not yet bearing flowers, transformation efficiencies of ~65-75% can be achieved (**Table 7**). Occasionally, whitish calli are formed on the side of an explant that is not in contact with the medium and, therefore, do not experience kanamycin selection. These calli are often not transgenic and any shoots formed from these calli will generally bleach and die after direct contact with kanamycin-containing medium (**Figure 2E**). In case the transformation rate is low and/or the starting material was suboptimal, tissue pieces might turn brown (**Figure 2F**) and suffer from over-proliferation by *A. tumefaciens* (**Figure 2G**). To prevent *A. tumefaciens* from spreading and overgrowing nearby explants, regular refreshment of the medium is required, and severely infected explants need to be removed. Once individual transgenic shoots are placed in the propagation medium, over-proliferation by *A. tumefaciens* is generally not occurring anymore (**Figure 2H**). Transgenic shoots can be multiplied through in vitro propagation, which will give rise to tens of shoots in a period of one month (**Figure 3A-B**). These shoots can be placed on rooting medium, which should induce root formation after ~2 weeks (**Figure 3C-D**). Rooted plantlets can be subsequently used for experimentation.

To create knockout mutant lines, we make use of CRISPR/Cas9-mediated mutagenesis. To this end, we make use of a binary vector containing the kanamycin resistance gene *NPTII*, a Cas9-encoding sequence driven by the CaMV35S promoter and 2 sgRNAs per target gene that are expressed from the AtU6p small RNA promoter[20]. A graphical representation of the construct used for CRISPR/Cas9-mediated mutagenesis of *P. andersonii* is provided in **Figure 4A**. Using this method, genome editing is observed in ~40% of putatively-transformed shoots[10]. To identify mutant lines, putatively-transformed shoots are genotyped for mutations at the sgRNA target site(s) using primers spanning the targeted region. An example of the expected results is given in **Figure 4**. As can be seen from the photo taken after gel electrophoresis, several samples produce a PCR amplicon with similar size to the wild type (**Figure 4B**). These plants may contain small indels that cannot be visualized by agarose gel electrophoresis or remain unedited by the Cas9 enzyme. Additionally, several samples yield bands that are different in size from the wild type (e.g., lines 2, 4, 7 and 8 in **Figure 4B**). In these lines, 1 (lines 4, 7 and 8) or both (line 2) alleles contain larger indels that can be easily visualized. The exact nature of the mutations at the target site(s) is revealed after PCR amplicon sequencing. As can be seen from **Figure 4C**, both small indels of 1-4 bp, as well as, larger deletions can be obtained after CRISPR/Cas9 mutagenesis. In **Figure 4C**, the sequence of line 1 is identical to that of the wild type, indicating that this line escaped editing and, therefore, should be discarded. Among the lines that contain mutations, heterozygous, homozygous and bi-allelic mutants can be identified (**Figure 4C**). However, heterozygous mutants are generally rare[10]. Homozygous or bi-allelic knockout mutants can be propagated vegetatively to obtain sufficient material for phenotypic analysis. As phenotypic analysis is performed in the T_0 generation, it is important to check whether mutant lines might be chimeric. To this end, genotyping needs to be repeated on at least 3 different samples taken from each mutant line. If the genotyping results are identical to each other and the original genotyping sample (e.g., line 8 in **Figure 4D**), the line is homogeneously mutated and can be used for further analysis. However, if the genotyping results differ between independent samples (e.g., line 4 in **Figure 4D**), the mutant line is chimeric and needs to be discarded.

Inoculation of *P. andersonii* with *M. plurifarium* BOR2 results in the formation of root nodules (**Figure 5**). As can be seen in **Figure 5A**, these nodules are distributed along the root system. Nodules of *P. andersonii* are light brown in color but can be easily discriminated from the root tissue based on their shape (**Figure 5B**). Inoculation experiments in pots and subsequent growth for 4-6 weeks typically result in the formation of ~10-30 nodules (**Figure 6A**). A similar number of nodules is formed after inoculation of EKM plate-grown *P. andersonii* plantlets at 4 weeks after inoculation (**Figure 6A**). In pouches, *P. andersonii* seedlings typically form ~5-15 nodules at 5 weeks post inoculation (**Figure 5C-D, 6A**). To analyze the nodule cytoarchitecture, nodules can be sectioned and observed using bright-field microscopy. **Figure 6B** shows an example of a longitudinal section through the middle of a *P. andersonii* nodule. This section shows the central vascular bundle of a *P. andersonii* nodule, which is flanked by nodule lobes containing infected cells (**Figure 6B**).

P. andersonii plantlets can also be mycorrhized. After 6 weeks of inoculation with *R. irregularis*, mycorrhizal colonization frequency typically reaches > 80% (**Figure 6C**). At this time point, generally ~30% of the cells contain arbuscules (**Figure 6C**). A representative image of a *P. andersonii* root segment containing arbuscles is shown in **Figure 6D**.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative images of a *P. andersonii* tree, seeds and seedlings. (**A**) Six-month old *P. andersonii* tree grown in potting soil in a greenhouse conditioned at 28 °C. (**B**) Representative image depicting *P. andersonii* berries at various stages of maturation. Young *P. andersonii* berries (unripe) will change color from green to white and finally to brown (ripe) upon ripening. (**C**) *P. andersonii* seeds incubated on SH-0 medium for 1 week. A black circle indicates a germinated seedling. (**D**) Four-week old *P. andersonii* seedlings grown in SH-0 medium. Scale bars are equal to 25 cm in (**A**) and 1 cm in (**B-D**).

Figure 2: Representative images of explants at different stages of the stable transformation procedure. (**A**) Explant co-cultivated with *A. tumefaciens*. (**B**) Explant overgrown by *A. tumefaciens* during the first 2 weeks post transformation. (**C**) Transgenic micro-callus formed near the wound site of an explant at 2.5 weeks post co-cultivation. (**D**) Representative image of an explant at 6 weeks post co-cultivation showing the emergence of shoots from (transgenic) calli. (**E**) Representative image of a shoot that becomes whitish and eventually dies when in direct contact with kanamycin-containing medium. This shoot is most likely non-transgenic and escaped kanamycin selection when attached to the explant. (**F**) Representative image of an unsuccessfully transformed explant. (**G**) Representative image of an unsuccessfully transformed explant overgrown by *A. tumefaciens*. (**H**) Single transgenic shoot grown on propagation medium at 8 weeks post co-cultivation with *A. tumefaciens*. Scale bars equal 2.5 mm. Boxes containing green check marks or red crosses indicate successful or unsuccessful transformation of explants, respectively.

Figure 3: Representative images of *in vitro* propagation. (**A**) Shoots grown on propagation medium. The image was taken 1 week after plates were refreshed. (**B**) Shoots grown on

propagation medium. The image was taken 4 weeks after plates were refreshed. (**C**) Freshly cut shoots placed on rooting medium. (**D**) Shoots incubated on rooting medium for 2 weeks. Note the presence of roots. Scale bars are equal to 2.5 cm.

Figure 4: Representative results after genotyping of *P. andersonii* T_0 transgenic CRISPR/Cas9 mutant lines. (**A**) Representative map of a binary vector used for CRISPR/Cas9-mediated mutagenesis of *P. andersonii*. (**B**) Representative result after PCR-based genotyping of potential CRISPR/Cas9 mutant lines using primers spanning the sgRNA target site(s). Shown is an image after agarose gel electrophoresis of amplicons. Samples taken from individual transgenic lines are indicated by numbers. Wild type (WT) and no template control (NTC) indicate lanes containing positive and negative controls, respectively. (**C**) Schematic representation of mutant alleles obtained after CRISPR/Cas9-mediated gene editing. Highlighted in blue and red colors are the sgRNA target sites and PAM sequences, respectively. (**D**) Representative result after PCR-based screening for potential chimeric mutant lines. Shown is an image after agarose gel electrophoresis of 3 individual samples taken from mutant lines 4 and 8. Note that transgenic mutant line 4 is chimeric.

Figure 5: Representative images of nodulation assays in plates and pouches. (**A**) Nodulation on plates containing agar-solidified EKM medium and inoculated with *M. plurifarium* BOR2 for 4 weeks. (**B**) Representative image of a *P. andersonii* root nodule. The image was taken at 4 weeks post inoculation with *M. plurifarium* BOR2. (**C**) Nodulation in pouches containing liquid EKM medium. Seedlings were inoculated with *Bradyrhizobium* sp. Kelud2A4 for 5 weeks. (**D**) Representative image of a complete setup used for the nodulation in pouches. Scale bars are equal to 2.5 cm in (**A,C**), 1 mm in (**B**), and 5 cm in (**D**).

Figure 6: Representative results of the nodulation and mycorrhization assays. (**A**) Representative bar graph showing the number of nodules formed per plant at 4 weeks post inoculation with *M. plurifarium* BOR2 in pots or on plates and at 5 weeks post inoculation with *Bradyrhizobium* sp. Kelud2A4 in pouches. Data represent mean ± SD (n = 10). (**B**) Representative image of a longitudinal section through a nodule formed at 4 weeks post inoculation with *M. plurifarium* BOR2. The section is stained with toluidine blue. (**C**) Representative bar graph showing quantification of mycorrhization. Variables quantified according to Trouvelot et al.[29] are F, the frequency of analyzed root fragments that are mycorrhized; M, the intensity of infection; A, the abundance of mature arbuscules in the total root system. Mycorrhization was quantified at 6 weeks post inoculation with *R. irregularis* (strain DAOM197198). Data represent mean ± SD (n = 10). (**D**) Representative image of mature arbuscules present in *P. andersonii* root cortical cells at 6 weeks post inoculation with *R. irregularis*. Scale bars equal 75 µm.

Table 1: Composition of Schenk-Hildebrandt-based[30] media used for growing *P. andersonii* seedlings, stable transformation, and *in vitro* propagation.

Dissolve solid compounds into 750 mL of ultra-pure water before adding liquid stocks. Afterwards, fill the complete medium to 1 L. Prepare BAP, IBA, NAA stocks in 0.1 M KOH and store at -20 °C.

Table 2: Composition of Yeast-Mannitol (YEM) medium used for growing rhizobium.
Adjust the pH to 7.0 and fill with ultra-pure water to 1 L. To prepare the agar-solidified YEM medium, add 15 g of microagar before autoclaving.

Table 3: Composition of 1 L modified EKM medium[31] used for *P. andersonii* nodulation assay.
The composition of the 500x micro-elements stock solution is listed in Table 4. To prepare 2% agar-solidified EKM medium, add 20 g of Daishin agar before autoclaving. Autoclave the $MgSO_4 \cdot 7H_2O$, Na_2SO_4, $CaCl_2 \cdot 2H_2O$, and Fe(III)-citrate stocks to sterilize. Filter sterilize NH_4NO_3 stock solution to sterilize.

Table 4: Composition of the 500x micro-elements stock solution used for preparing modified EKM medium. Store the micro-elements stock solution at 4 °C.

Table 5: Composition of ½-Hoagland[32] medium used for mycorrhization assays. The composition of the 50x micro-elements stock solution is listed in Table 6. Prepare the Fe(II)-EDTA solution by combining $FeSO_4 \cdot 7H_2O$ (9 mM) and $Na_2 \cdot$EDTA (9 mM) into 1 stock solution, and store at 4 °C. Adjust the pH of the medium to 6.1 using 1 M KOH and fill with ultra-pure water to 1 L.

Table 6: Composition of the 50x micro-elements stock solution used for preparing ½-Hoagland medium.

Table 7: Transformation efficiency of *P. andersonii*. Here, transformation efficiency is defined as the percentage of explants that form at least 1 transgenic callus or shoot. Transformation efficiency was scored at 6 weeks post transformation and is depicted as mean ± SD. n indicates the number of transformation experiments from which the transformation efficiency was determined.

Supplemental File 1: Overview of level 1 and level 2 constructs used for CRISPR/Cas9 mutagenesis.

DISCUSSION:
Legumes and the distantly-related Cannabaceae genus *Parasponia* represent the only two clades of plant species able to establish an endosymbiotic relationship with nitrogen-fixing rhizobia and form root nodules. Comparative studies between species of both clades are highly relevant to provide insights into the core genetic networks allowing this symbiosis. Currently, genetic studies are mainly done in legumes; especially the two model species *M. truncatula* and *L. japonicus*. To provide an additional experimental platform and facilitate comparative studies with a nodulating non-legume, we describe here a detailed protocol for stable transformation and reverse genetic analyses in *P. andersonii*. The presented protocol uses in vitro propagation of T_0 transgenic *P. andersonii* lines, allowing phenotypic analysis to be initiated within 4 months after *A. tumefaciens* co-cultivation. This is substantially faster than current protocols that have been established for stable transformation of legumes[33]. This makes *P. andersonii* an attractive research model.

The protocol described here contains several critical steps. The first of which concerns seed

749 germination. To prepare *P. andersonii* seeds for germination, seeds need to be isolated from the
750 berries. This is done by rubbing the berries on a piece of tissue paper or against the inside of a
751 tea sieve. This procedure needs to be performed gently in order to prevent damage to the seed
752 coat. If the seed coat gets damaged, bleach could enter the seed during sterilization, which
753 reduces seed viability. To break seed dormancy, seeds are subjected to a 10-day temperature
754 cycle. However, despite this treatment, germination is not entirely synchronized. Generally, the
755 first seeds show radicle emergence after 7 days, but others might take several days longer to
756 germinate.
757
758 Critical points in the transformation procedure concern the choice of the starting material and
759 the duration of the co-cultivation step. To reach efficient transformation, it is best to use healthy
760 and young stems or petioles of non-sterile greenhouse-grown plants as the starting material. In
761 order to induce the growth of young branches, it is advisable to trim *Parasponia* trees every 2-3
762 months and refresh trees once a year. Additionally, the co-cultivation step needs to be performed
763 for 2 days only. Prolonged co-cultivation promotes over-colonization of tissue explants by *A.*
764 *tumefaciens* and generally reduces transformation efficiency. To prevent over-colonization by *A.*
765 *tumefaciens* it is also important to regularly refresh the plates on which the explants are
766 cultivated. In case over-colonization does occur, tissue explants could be washed (see Section
767 3.8) to remove *A. tumefaciens* cells. We advise adding bleach to the SH-10 solution used for
768 washing (final concentration: ~2% hypochlorite). It is important to note that this additional
769 washing step might not work on heavily-infected explants (**Figure 2B**). In case a transformation
770 with a CRISPR/Cas9 construct yields only a limited number of putatively-transformed shoots or if
771 mutagenesis of a particular gene is expected to cause problems in regeneration, it is advisable to
772 include an empty vector control construct as the positive control. Lastly, it is important to ensure
773 that all transgenic lines that are selected are resulting from independent T-DNA integration
774 events. Therefore, we instruct to take only a single putatively-transgenic shoot from each side of
775 an explant. However, we realize that this reduces the potential number of independent lines. If
776 many lines are required, researchers could decide to separate putatively-transformed calli from
777 the original explants when these calli are ≥2 mm in size and culture these calli independently. In
778 this way, multiple lines could be isolated from each explant, which raises the number of potential
779 transgenic lines.
780
781 In the current protocol, transgenic lines of *P. andersonii* are propagated vegetatively through in
782 vitro propagation. The advantage of this is that many transgenic plantlets can be generated in a
783 relatively short time period. However, this method also has several limitations. Firstly, the
784 maintenance of T_0 transgenic lines through in vitro propagation is labor intensive and could result
785 in unwanted genetic or epigenetic alterations[34,35]. Secondly, T_0 lines still contain a copy of the T-
786 DNA, including the antibiotic resistance cassette. This limits the number of possible re-
787 transformations, as different selection markers are required for each re-transformation.
788 Currently, we have only tested transformation using kanamycin or hygromycin selection (data
789 not shown). Furthermore, the presence of the Cas9-encoding sequence and sgRNAs in the T_0
790 transgenic lines complicates complementation studies. Complementation assays are possible but
791 require the sgRNA target site(s) to be mutated as such that gene-editing of the complementation
792 construct is prevented. Thirdly, a disadvantage of working with T_0 lines is that CRISPR/Cas9

mutants might be chimeric. To prevent phenotypic analysis of chimeric mutant lines, we recommend repeating the genotyping analysis after in vitro propagation on at least 3 different shoots. Although, the number of chimeric mutants obtained using the protocol described here is limited, they are occasionally observed[10]. To overcome the limitations of working with T_0 lines, *P. andersonii* mutant lines could be propagated generatively. *P. andersonii* trees are dioecious and wind-pollinated[2]. This means that each transgenic line needs to be manipulated as such that male and female flowers are produced on a single individual, and subsequently grown as such that cross pollination does not occur. As *P. andersonii* is a fast-growing tree it requires a substantial amount of space in a tropical greenhouse (28 °C, ~85% relative humidity). Therefore, although technically possible, generative propagation of *P. andersonii* transgenic lines is logistically challenging.

In the protocol section, we described 3 methods for nodulation of *P. andersonii*. The advantage of the plate and pouch systems is that the roots are easily accessible, which may allow spot-inoculation of bacteria and following nodule formation over time. However, the plate system is quite labor intensive, which makes it less suited for large-scale nodulation experiments. A disadvantage of the pouch system is that it is difficult to prevent fungal contamination. Pouches are not sterile, and therefore fungal growth is often observed on the top half of the pouch. However, this does not affect *P. andersonii* growth, and therefore does not interfere with nodulation assays. Additionally, the pouch system is only suitable for seedlings. Despite several attempts, we have been unable to grow plantlets obtained through in vitro propagation in pouches.

The *P. andersonii* reverse genetics pipeline described here offers a substantial improvement compared to the existing *A. rhizogenes*-based root transformation method[11]. Using the described procedures, stable transgenic lines can be generated efficiently and can be maintained via in vitro propagation. In contrast, *A. rhizogenes* transformation is transient and only results in the formation of transgenic roots. Because each transgenic root results from an independent transformation, *A. rhizogenes*- transformation-based assays suffer from substantial phenotypic variation. This variation is much less in case of stable lines, although in vitro propagation also creates some level of variation. Because of this reduced variation and the fact that multiple plantlets could be phenotyped for each stable line, stable lines are more suited for quantitative assays compared to *A. rhizogenes*-transformed roots. Additionally, the stable transformation does not depend on the introduction of the *A. rhizogenes root inducing locus* (*rol*) that affects the endogenous hormone balance[15]. Therefore, stable lines are better suited for reverse genetic analysis of genes involved in hormone homeostasis compared to *A. rhizogenes*-transformed roots. A more general advantage of *P. andersonii* as research model is that it did not experience a recent whole genome duplication (WGD). The legume Papilionoideae subfamily, which includes the model legumes *M. truncatula* and *L. japonicus*, as well as the Salicaceae (order Malpighiales) that includes the model tree *Populus trichocarpa* experienced WGDs ~65 million years ago[36,37]. Many paralogous gene copies resulting from these WGDs are retained in the genomes of *M. truncatula*, *L. japonicus* and *P. trichocarpa*[37–39], which creates redundancy that might complicate reverse genetic analyses. As *P. andersonii* did not experience a recent WGD, reverse genetic analyses on *P. andersonii* might be less affected by redundant functioning of paralogous gene

copies.

Taken together, we provide a detailed protocol for reverse genetic analysis in *P. andersonii*. Using this protocol, single mutant lines can be efficiently generated in a timeframe of 2-3 months[10]. This protocol can be extended to create higher order mutants through multiplexing of sgRNAs targeting different genes simultaneously, as shown for other plant species[40–42]. Additionally, the stable transformation procedure described here is not limited to CRISPR/Cas9 gene-targeting but could also be used to introduce other types of constructs (e.g., for promoter-reporter assays, ectopic expression or *trans*-complementation). We established *P. andersonii* as a comparative research model to study mutualistic symbioses with nitrogen-fixing rhizobia or endomycorrhizal fungi. However, the protocols described here also provide tools to study other aspects of the biology of this tropical tree, such as wood formation, the development of bi-sexual flowers or the biosynthesis of Cannabaceae-specific secondary metabolites.

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DISCLOSURES:
The authors have nothing to disclose.

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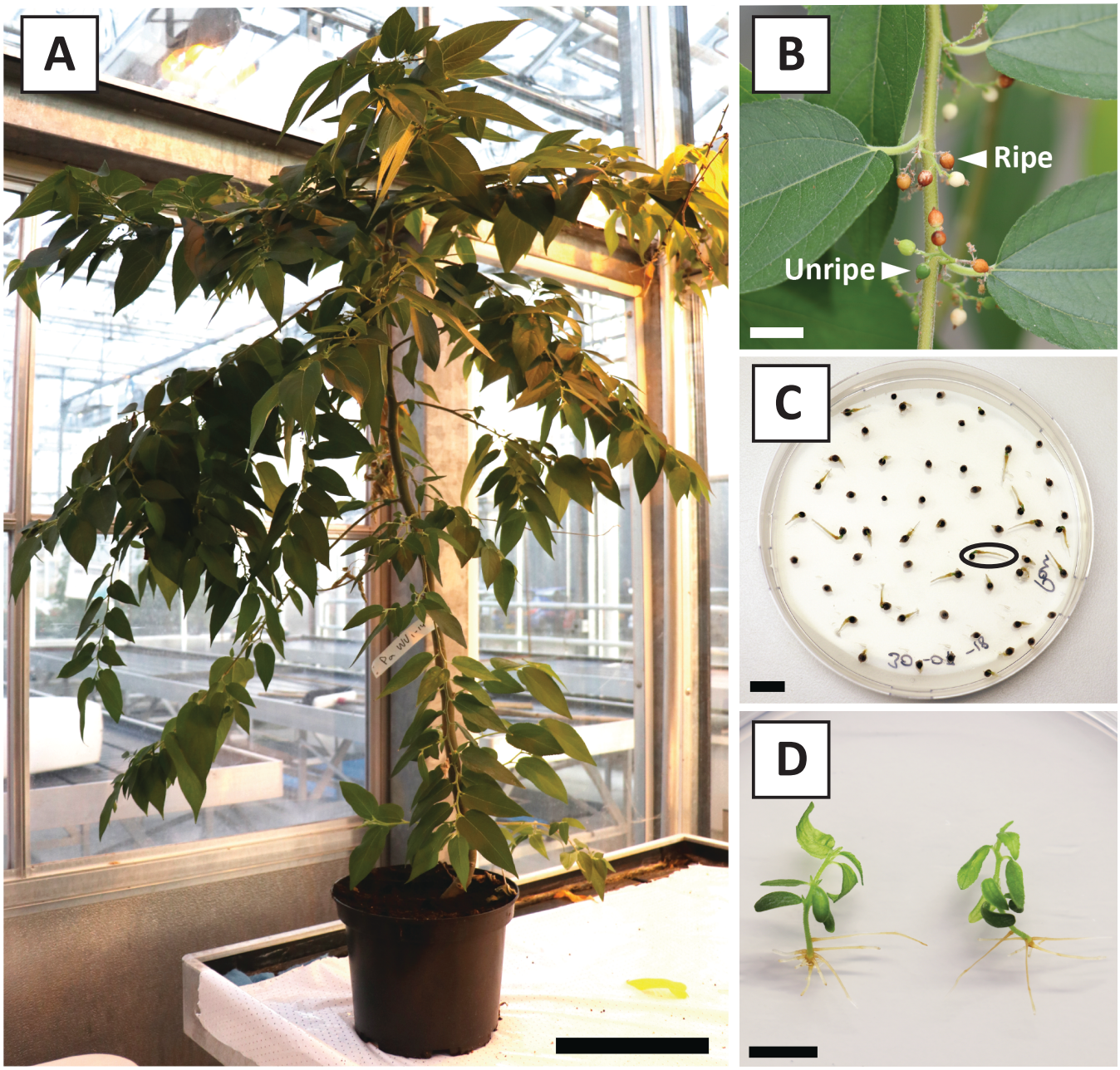

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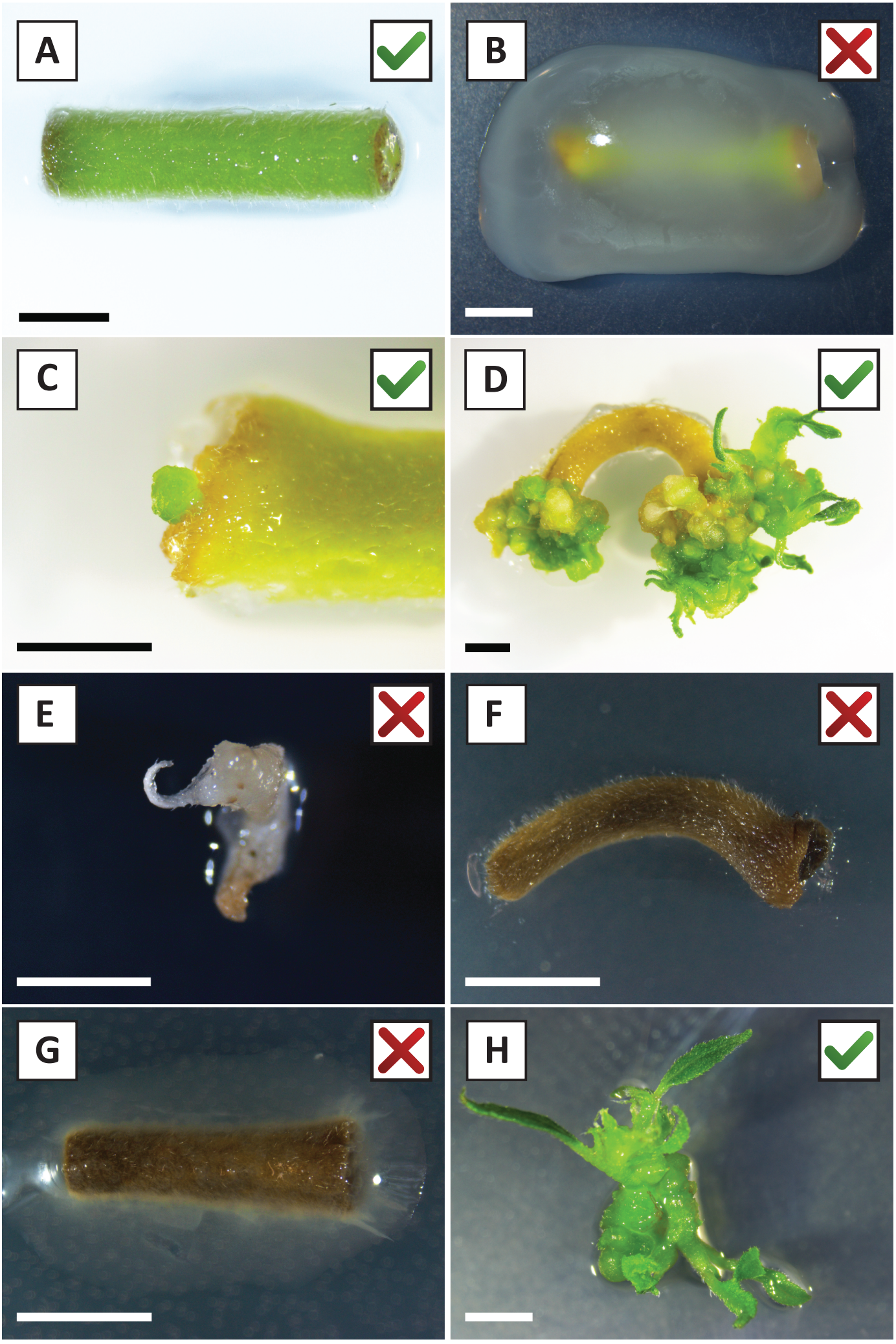

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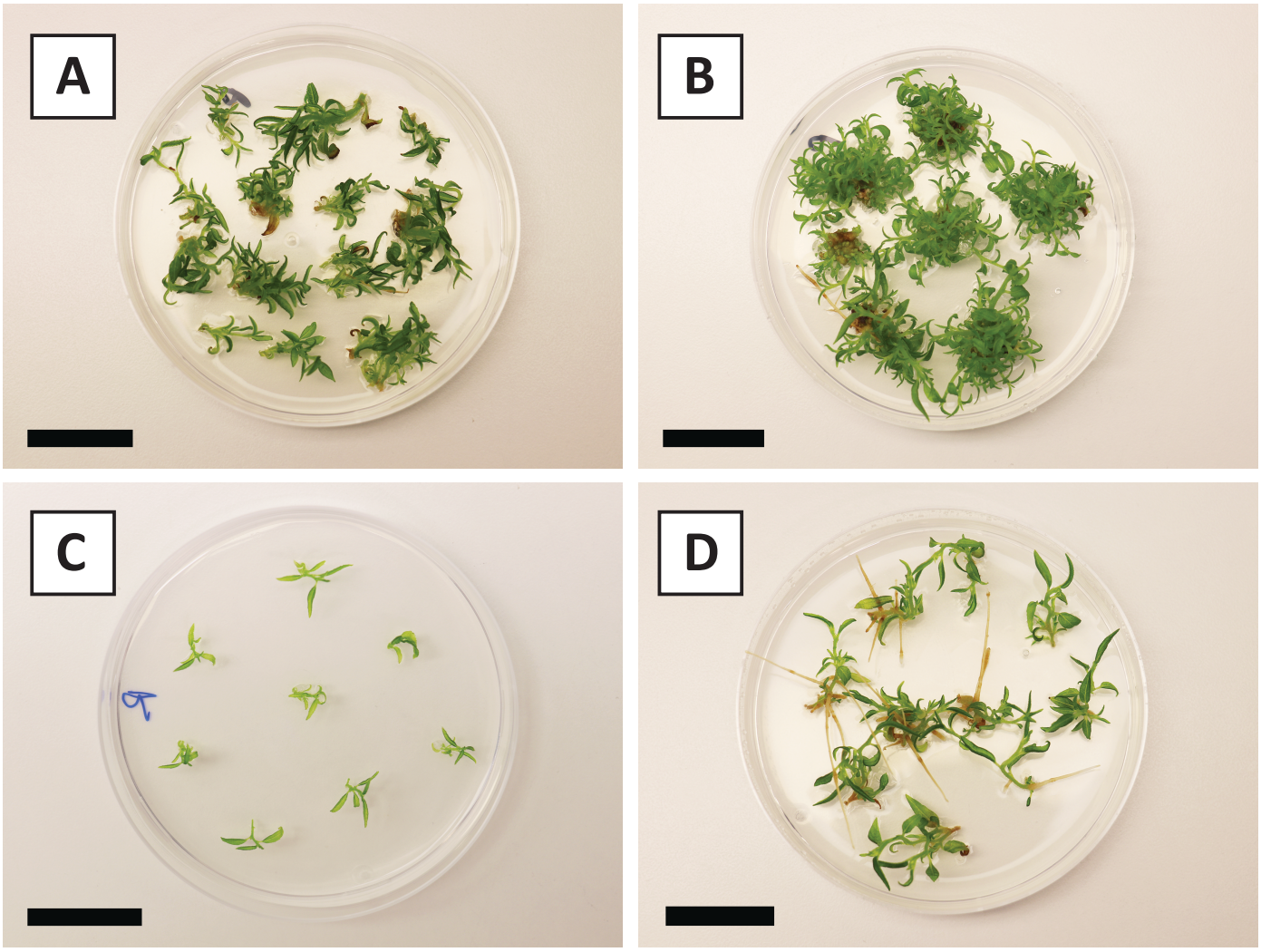

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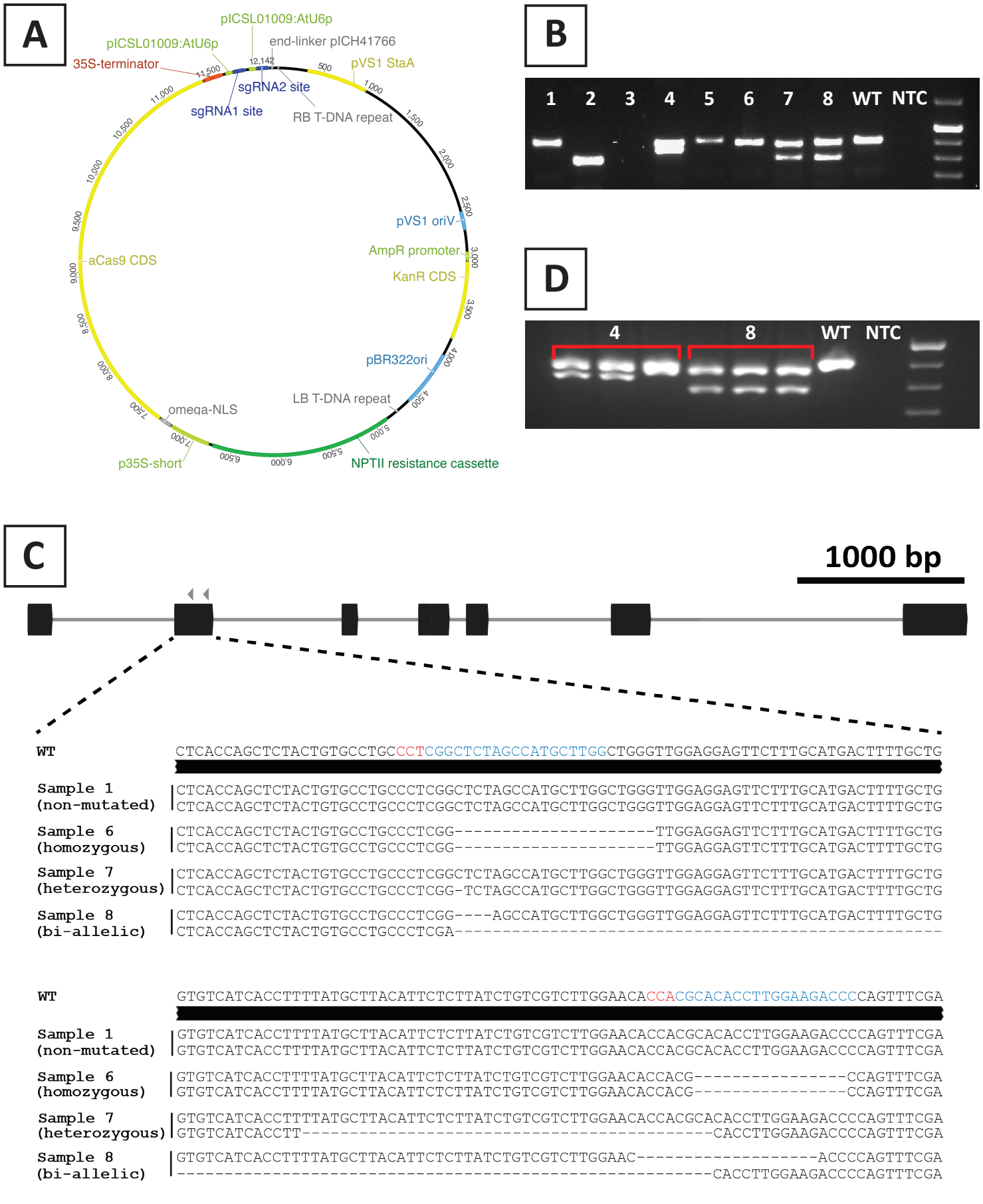

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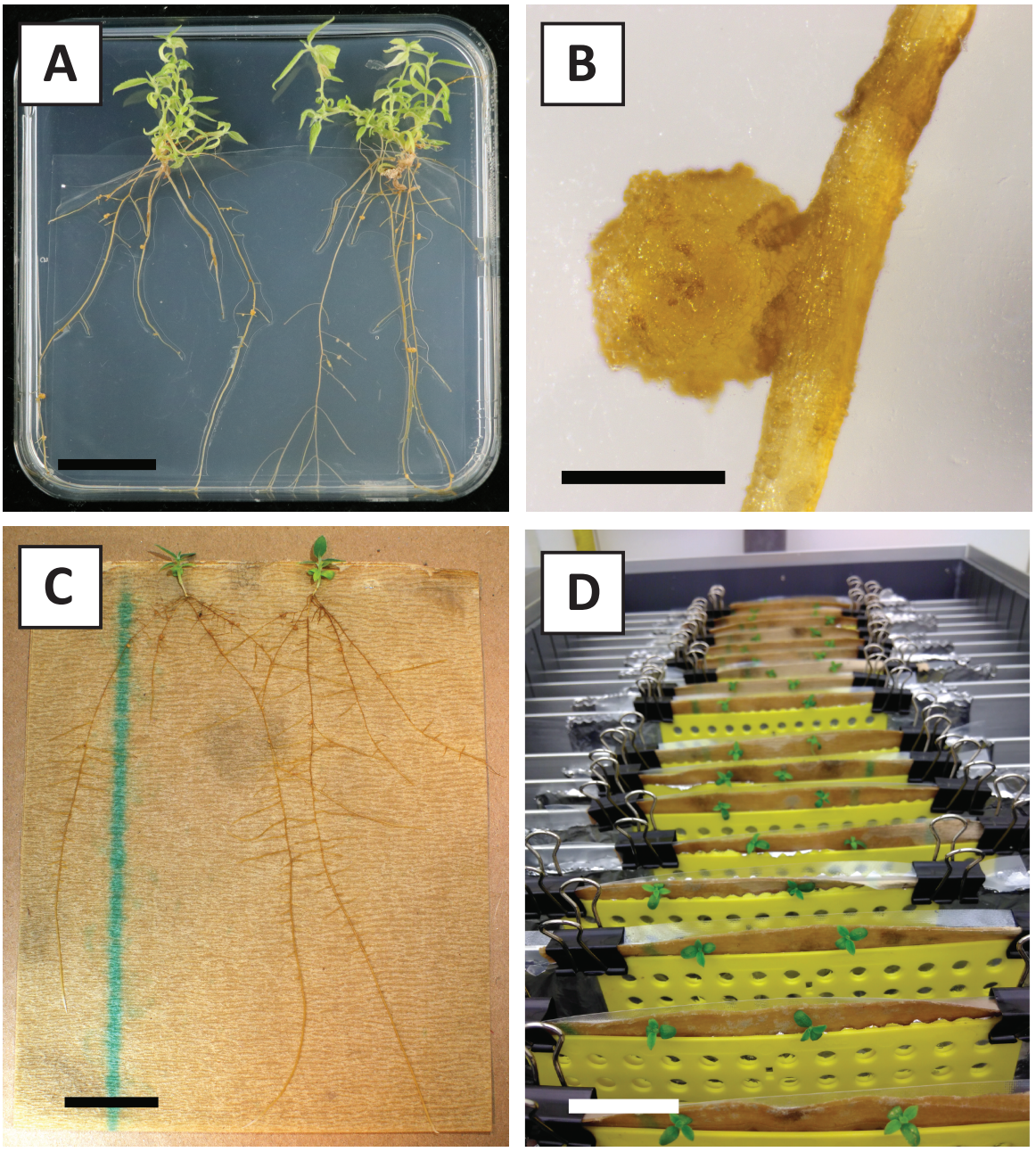

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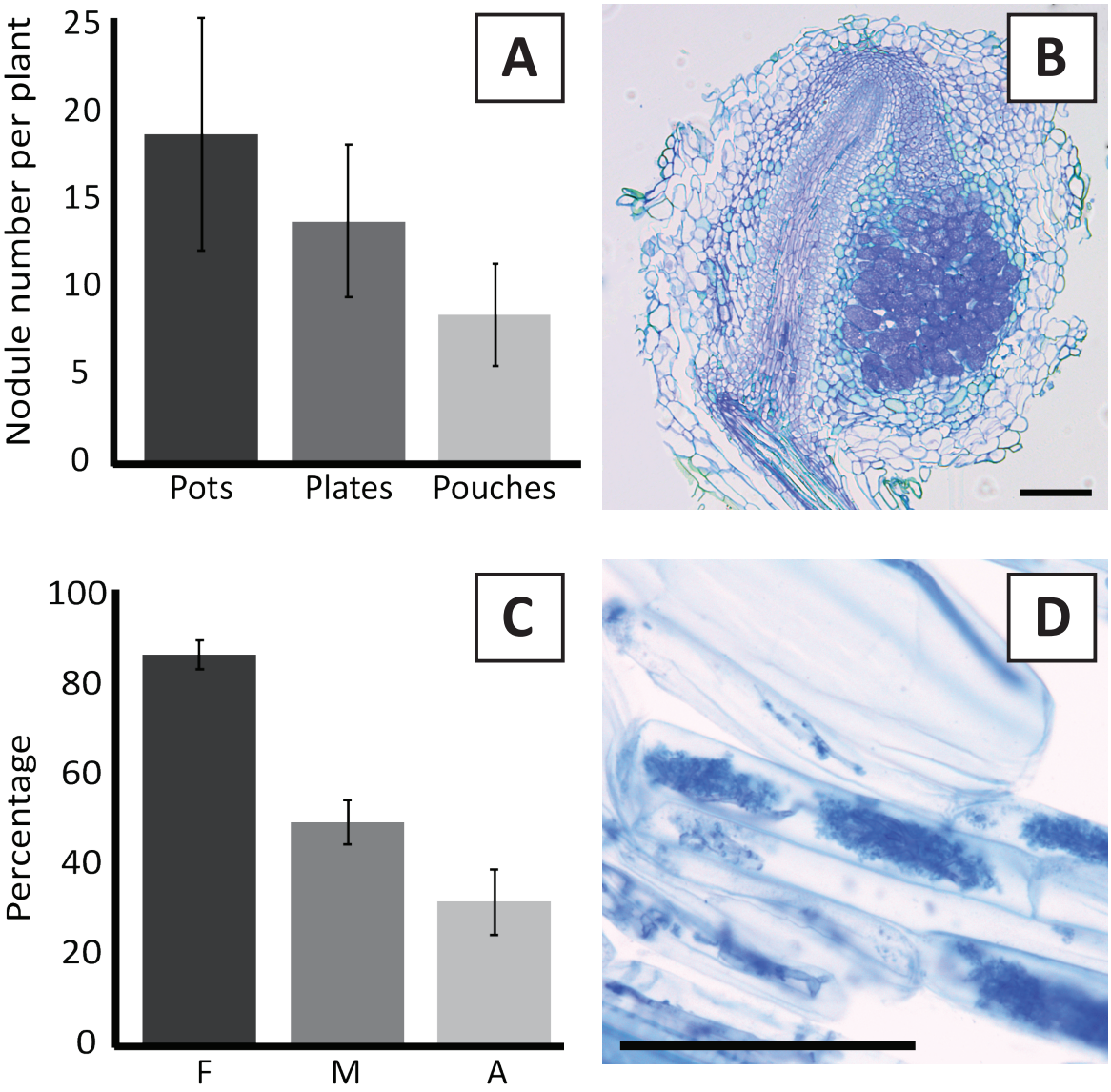

Table 1

Compound	SH-0	SH-10	Propagation medium	Rooting medium	Infiltration medium
SH-basal salt medium	3.2 g	3.2 g	3.2 g	3.2 g	3.2 g
SH-vitamin mixture	1 g	1 g	1 g	1 g	1 g
Sucrose	-	10 g	20 g	10 g	10 g
BAP (1 mg/mL)	-	-	1 mL (4.44 µM)	-	-
IBA (1 mg/mL)	-	-	100 µL (0.49 µM)	1 mL (4.92 µM)	-
NAA (1 mg/mL)	-	-	-	100 µL (0.54 µM)	-
1 M MES pH=5.8	3 mL	3 mL	3 mL	3 mL	3 mL
1 M KOH	Adjust pH to 5.8	Adjust pH to 5.8	Adjust pH to 5.8	Adjust pH to 5.8	Adjust pH to 5.8
Daishin agar	8 g	-	8 g	8 g	-

Before autoclaving:		
Compound	**Amount per liter**	**Final concentration**
Mannitol	5 g	27.45 mM
Na-Gluconate	5 g	22.92 mM
Yeast extract	0.5 g	-
$MgSO_4 \cdot 7H_2O$	0.2 g	0.81 mM
NaCl	0.1 g	1.71 mM
K_2HPO_4	0.5 g	2.87 mM
After autoclaving:		
Compound	**Amount per liter**	**Final concentration**
1.5 M $CaCl_2$	1 mL	1.5 mM

Table 3

Before autoclaving:			
Compound	**Stock concentration**	**Amount per liter medium**	**Final concentration**
KH_2PO_4	0.44 M	Add 2 mL	0.88 mM
K_2HPO_4	1.03 M	Add 2 mL	2.07 mM
500x micro-elements stock solution	-	Add 2 mL	-
MES pH=6.6	1 M	Add 3 mL	3 mM
HCl	1 M	Adjust pH to 6.6	-
Ultra-pure water	-	Fill to 990 mL	-
After autoclaving:			
Compound	**Stock concentration**	**Amount per liter medium**	**Final concentration**
$MgSO_4 \cdot 7H_2O$	1.04 M	2 mL	2.08 mM
Na_2SO_4	0.35 M	2 mL	0.70 mM
NH_4NO_3	0.18 M	2 mL	0.36 mM
$CaCl_2 \cdot 2H_2O$	0.75 M	2 mL	1.5 mM
Fe(III)-citrate	27 mM	2 mL	54 µM

Table 4

Compound	Amount per liter	Stock concentration
$MnSO_4$	500 mg	3.31 mM
$ZnSO_4 \cdot 7H_2O$	125 mg	0.43 mM
$CuSO_4 \cdot 5H_2O$	125 mg	0.83 mM
H_3BO_3	125 mg	2.02 mM
$Na_2MoO_4 \cdot 2H_2O$	50 mg	0.21 mM

Table 5

Compounds	Stock concentration	Amount per liter medium	Final concentration
K_2HPO_4	20 mM	1 mL	0.2 mM
NH_4NO_3	0.28 M	10 mL	2.8 mM
$MgSO_4$	40 mM	10 mL	0.4 mM
K_2SO_4	40 mM	10 mL	0.4 mM
Fe(II)-EDTA	9 mM	10 mL	0.9 mM
$CaCl_2$	80 mM	10 mL	0.8 mM
50x micro-elements stock solution	-	10 mL	-

Table 6

Compounds	Amount per liter	Stock concentration
H_3BO_3	71.1 mg	1.15 mM
$MnCl_2 \cdot 4H_2O$	44.5 mg	0.22 mM
$CuSO_4 \cdot 5H_2O$	3.7 mg	23.18 µM
$ZnCl_2$	10.2 mg	74.84 µM
$Na_2MoO_4 \cdot 2H_2O$	1.2 mg	4.96 µM

Table 7

Age of explants	Transformation efficiency
Young	69.4 ± 6.2% (n = 2)
Mature	18.3 ± 10.2% (n = 15)

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1-Naphthaleneacetic acid	Sigma-Aldrich	N0640	NAA
2-(N-morpholino)ethanesulfonic acid	Duchefa Biochemie	M1503.0250	MES
3',5'-Dimethoxy-4'-hydroxyacetophenone	Sigma-Aldrich	D134406	Acetosyringone
5-Bromo-3-indolyl β-D-galactopyranoside	Duchefa Biochemie	X1402.1000	X-Gal
Agarose	Merck	101236	For nucleic acid electrophoresis gel
Aluminium AW-6060 flat 50x2mm	-	-	Pouches box material, hangers
Ammonium nitrate	Merck	101188	NH_4NO_3
Benzylaminopurine	Sigma-Aldrich	B3408-1G	BAP
Boric acid	Merck	100156	H_3BO_3
BpiI (BbsI) (10 U/µL)	Thermo-Fisher	ER1011	Used as restriction enzyme in Golden Gate cloning assembly
BSA (Bovine Serum Albumin)	Thermo-Fisher	15561020	Used in Golden Gate cloning assembly
Calcium chloride dihydrate	Merck	137101	$CaCl_2 \cdot 2H_2O$
Cefotaxime sodium	Duchefa Biochemie	C0111.0025	$C_{16}H_{16}N_5O_7S_2Na$
CloneJET PCR Cloning Kit	Thermo-Fisher	K1231	Used for cloning the blunt-ended PCR amplicons in genotyping procedure
CONNECTIS Agronutrition	Agronutrition	AP2011	Containing *Rhizophagus irregularis* DAOM 197198 (1,000 spores/mL), used for mychorrization assay
Copper(II) sulfate pentahydrate	Merck	102790	$CuSO_4 \cdot 5H_2O$
Daishin agar	Duchefa Biochemie	D1004.1000	Used for plant tissue culture agar-based medium
Dipotassium hydrogen phosphate	Merck	105101	K_2HPO_4
Disodium ethylenediaminetetraacetate dihydrate	VWR Chemicals	20302.293	Na_2·EDTA
D-Mannitol	Duchefa Biochemie	M0803.1000	$C_6H_{14}O_6$
Eco31I (BsaI) (10 U/µL)	Thermo-Fisher	ER0291	Used as restriction enzyme in Golden Gate cloning assembly
Ethanol	Merck	100983	C_2H_5OH
Ethylenediaminetetraacetic acid	VWR Chemicals	BDH9232-500G	EDTA
Gel drying frames 24 cm x 24 cm	Sigma-Aldrich	Z377600-1PAK	Cellophane membrane
Geneious	Biomatters, Ltd.	R9 or higher	Bioinformatics software for *in silico* cloning and designing of sgRNAs
Germination pouches (16.5x17.5 cm)	Mega International	-	Technical information at https://mega-international.com/tech-info/
Glutaraldehyde 25%	Sigma-Aldrich	65882	Used for fixating nodule tissues
Glycerol	VWR Chemicals	24385.295	-
Grey PVC 5mm thick	Vink	219341	Pouches box material, bottom part
HistoMold disposable polyethylene mold (size 6x8mm)	Leica Biosystems	14702218311	Used as a template for plastic embedding
Hydrochloric acid fuming 37%	Merck	100317	HCl
Indole-3-butyric acid	Sigma-Aldrich	I5386-1G	IBA
Iron(II) citrate	Merck	103862	$C_6H_5FeO_7$
Iron(II) sulfate heptahydrate	Merck	103965	$FeSO_4 \cdot 7H_2O$
Isopropyl β-D-1-thiogalactopyranoside	Duchefa Biochemie	I1401.0005	IPTG
Kanamycine monosulfate	Duchefa Biochemie	K0126.0010	
Lactic acid	Sigma-Aldrich	L2000	
Magnesium sulfate	Merck	105886	$MgSO_4 \cdot 7H_2O$
Manganese(II) chloride	Merck	105934	$MnCl_2 \cdot 4H_2O$
Manganese(II) sulphate	Merck	102786	$MnSO_4$
Micro agar	Duchefa Biochemie	M1002.1000	Used for bacterial culture agar-based medium
Paper clips 19mm	Manutan	92007687	Pouches material
Parafilm "M"	Paraxisdienst	130774	Elastic sealing foil
Perlite size no.3	Pull Rhenen	Agra-Perlite No.3	Used as growing substrate in pots for nodulation assay
Petri dishes with cover (Borosilicate 3.3, size 150x25 mm)	VWR Chemicals	391-0581	Used as container for cellophane membranes
Phire Plant Direct PCR kit	Thermo-Fisher	F130WH	For genotyping transgenic lines
pICH41414	Addgene	50337	Level 0 terminator, 3'UTR, 35s (Cauliflower Mosaic Virus)
pICH41744	Addgene	48017	End-link 2 for assembling 2 level one part into a level 2 acceptor
pICH41766	Addgene	48018	End-link 3 for assembling 3 level one part into a level 2 acceptor
pICH47742	Addgene	48001	Level 1 acceptor. Position 5. Forward orientation
pICH47802	Addgene	48007	Level 1 Acceptor. Position 1. Reverse orientation
pICH51277	Addgene	50268	Level 0 promoter (0.4 kb), 35s (Cauliflower Mosaic Virus) + 5'UTR, Ω (Tobacco Mosaic Virus)
pICH86966::AtU6p::sgRNA_PDS	Addgene	46966	Used for designing CRISPR/Cas9 module
pICSL01009::AtU6p	Addgene	46968	Used for designing CRISPR/Cas9 module
pICSL70004	Addgene	50334	Level 0 Kanamycin/Neomycin/Paromomycin resistance cassette
Polyester filters 210 µm, 120 µm and 36 µm mesh size	Topzeven	-	Used as filters for washing spore suspension
Polyethylene glycol 400	Sigma-Aldrich	8.17003	PEG400

Translucent polypropylene containers (OS140box)	Duchefa Biochemie	E1674.0001	Pots to grow *Parasponia* plantlets/seedlings
Potassium dihydrogen phosphate	Merck	104871	KH_2PO_4
Potassium hydroxide	Merck	105033	KOH
Potassium sulfate	Merck	105153	K_2SO_4
Sand, grain size 0.1-4 mm	Van Leusden b.v.	-	Used as growing substrate for mychorrhization assay
Schenk & Hildebrandt basal salt medium	Duchefa Biochemie	S0225.0050	SH-basal salt medium
Schenk & Hildebrandt vitamin mixture	Duchefa Biochemie	S0411.0250	SH-vitamin mixture
Silwet L-77	Lehle Seeds	VIS-02	Used as non-ionic surfactant in the washing step of stable transformation
Sodium chloride	Merck	137017	NaCl
Sodium gluconate	VWR Chemicals	89230-072	$C_6H_{11}NaO_7$
Sodium molybdate dihydrate	Merck	106521	$Na_2MoO_4 \cdot 2H_2O$
Sodium phosphate dibasic heptahydrate	Merck	106574	$Na_2HPO_4 \cdot 7H_2O$
Sodium phosphate monobasic monohydrate	Merck	567549	$NaH_2PO_4 \cdot H_2O$
Sodium sulfate	Sigma-Aldrich	239313	Na_2SO_4
Sucrose	Duchefa Biochemie	S0809.5000	$C_{12}H_{22}O_{11}$
T4 DNA Ligase Buffer (10X)	Thermo-Fisher	B69	Used in Golden Gate cloning assembly
T4 DNA Ligase, HC (30 U/µL)	Thermo-Fisher	EL0013	Used in Golden Gate cloning assembly
Technovit 3040 yellow powder	Kulzer-Mitsui Chemicals Group	64708806	Methyl methacrylate-based resin powder
Technovit 7100 Combipack (including Hardener I, Hardener II)	Kulzer-Mitsui Chemicals Group	64709003	HEMA (2-hydroxyethyl methacrylate)-based resin solution
Technovit universal liquid	Kulzer-Mitsui Chemicals Group	66022678	Methyl methacrylate-based resin solution
Toluidine blue	Merck	1159300025	
Trypan blue	Acros	189350250	
Tween-20	VWR Chemicals	663684B	Polysorbate 20
UV transparant Persplex 3mm thick	Stout Perspex	-	pouches box material, lid
Yeast extract	Duchefa Biochemie	Y1333.1000	
Zinc chloride	Merck	108816	$ZnCl_2$
Zinc sulfate heptahydrate	Alfa Aesar	33399	$ZnSO_4 \cdot 7H_2O$

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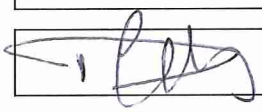
Signature:	
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Supplemental Table 1

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Construct	Description	Level	Backbone
1	*nptII* resistance casette	1	pICH47802
2	35Spro:ΩNLS-Cas9:35Ster	1	pICH47742
3	sgRNA1	1	pICH47751
4	sgRNA2	1	pICH47761
5	CRISPR/Cas9_ctrl	2	pICSL4723
6	CRISPR/Cas9_module	2	pICSL4723

Contains
pICSL70004:nptII
pICH41388:35Spro
pAGM5331:ΩNLS
pICH41308::aCas9
pICH41414:35Ster
pICSL01009:AtU6p, corresponding PCR amplicon
pICSL01009:AtU6p, corresponding PCR amplicon
1R: construct 1, 2F: construct 2, end-link pICH41744
1R: construct 1, 2F: construct 3, 3F: construct 4, end-link pICH41766

Reference
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Engler et al.[22]
Engler et al.[22]
Fauser et al.[23]
Engler et al.[22]
Nekrasov et al.[20]
Nekrasov et al.[20]
Engler et al.[22]
Engler et al.[22]