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## Supplemental Information

# Root Endophyte *Colletotrichum tofieldiae* Confers Plant Fitness Benefits that Are Phosphate Status Dependent

**Kei Hiruma, Nina Gerlach, Soledad Sacristán, Ryohei Thomas Nakano, Stéphane Hacquard, Barbara Kracher, Ulla Neumann, Diana Ramírez, Marcel Bucher, Richard J. O'Connell, and Paul Schulze-Lefert**

## Supplemental Experimental Procedures

### Detection of *C. tofieldiae* (*Ct*) from natural *A. thaliana* populations

Healthy *A. thaliana* leaves and roots from four different natural population sites in Spain were collected as described previously (García et al., 2013). Total DNA was isolated from the soil and the root samples using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, USA) and 10 ng of DNA template was used for PCR amplification using a specific primer pair that targets the *Ct* tubulin 2 sequence (CT04_11973), encompassing coding and intronic sequences. After 40 PCR cycles, the melting curves of these samples were compared with a control sample amplified from *Ct* genomic DNA. The tubulin primer sequences are AGTCTTTCCTGATCCCGACC and AAGTGGCCAGATCAAGTCAA.

### Fungal transformation and gene replacement

To visualize fungal hyphae surrounding or inside plant roots, the GFP gene under control of the constitutive GPDA promoter was introduced into *Ct* by *Agrobacterium*-mediated transformation using the binary plasmid pBin-GFP-hph as described previously (O'Connell et al., 2004). For targeted gene replacement, we used the binary plasmid pBIG4MRHrev carrying the hygromycin (Hyg) resistance cassette (Fukada and Kubo 2015). The In-Fusion HD Cloning Kit (Clontech) was used for plasmid construction. To generate replacement mutants lacking the acid phosphatase gene CT04_08450, a plasmid in which the Hyg resistance gene cassette was inserted between DNA sequences flanking the target gene were constructed. Approximately 1.5-kb fragments of 5' (5F) and 3' sequences (3F) flanking the CT04_08450 ORF were amplified from *Ct* genomic DNA. The purified PCR products 5F and 3F were mixed with *Sal*I digested pBIG4MRHrev for In-Fusion reactions, resulting in pBIG4MRHrev containing 5F-Hyg-3F. This plasmid was then introduced into *Ct* by *Agrobacterium*-mediated transformation. Hygromycin-resistant strains were tested by PCR to determine whether the acid phosphatase gene was successfully replaced by the Hyg resistance cassette (Figure S4). The PCR primers used are listed in Table S1.

### Microcopy

*A. thaliana* plants expressing aquaporin PIP2A fused with the fluorescent protein mCherry (Nelson et al., 2007) were grown in half-strength Murashige & Skoog (MS) agarose medium supplemented with 25 mM sucrose for two weeks and the roots were dip-inoculated with spores

of *Ct*-GFP (2-5 x $10^5$ spores/ml) for 5 minutes. The inoculated plants were then transferred to half-strength MS agarose medium without sucrose after washing them with distilled water (DW). Inoculated plants were grown at 22°C, with a 10-hour photoperiod (80 µE/$m^2$s), for 1 to 14 days. For visualization we used a Zeiss CLSM780 confocal microscope equipped with 10x and water-immersion x 63 objectives, using the 488-nm line of a 25-mW Argon ion laser for GFP and the 561-nm line of a 20-mW solid state laser for mCherry. Lambda mode (spectral imaging) was used to separate the RFP signal from autofluorescence, which can be used to identify boundaries of dead cells (i.e. the remaining cell wall structure). To track *Ct* hyphae in healthy leaves following root colonization, *A. thaliana* plants were grown hydroponically as described previously (Strehmel et al., 2014).

For light microscopy of resin-embedded sections, 2 mm segments of hydroponically grown *A. thaliana* roots infected with *Ct* were fixed in 2.5 % (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 6.9) overnight at 4 °C at 3 dpi, 5 dpi and 7 dpi. After washing in 0.05 M sodium cacodylate buffer, dehydration in a graded ethanol series and embedding in medium-grade LR White acrylic resin (Plano GmbH, Wetzlar, Germany) at 4 °C, samples were polymerized under UV light for 24 h at -20 °C and 24 h at 0 °C. For bright field observation, transverse semi-thin sections (1 µm) of infected roots were collected on glass slides, stained with 1 % (w/v) aqueous Toluidine blue supplemented with 1 % (w/v) sodium tetraborate, and viewed with a Zeiss Axioscope microscope fitted with an AxioCam Hrc camera. Images were processed using Adobe Photoshop software.

The same LR White-embedded material was also used for cytochemical detection of *N*-acetylglucosamine residues in fungal cell walls. For this purpose, semi-thin sections (1 µm) of infected *A. thaliana* roots were dried down on diagnostic adhesion slides (Thermo Fisher Scientific X2XER202W# AD CE) and incubated for 1 hour at room temperature in 5 % (v/v) goat normal serum in TRIS buffer (20 mM TRIS, 225 mM NaCl, pH 6.9) supplemented with 1 % (w/v) BSA (TRIS-BSA). After washing (10 min x 3) in TRIS-BSA, sections were incubated in a 1:10 dilution of wheat germ agglutinin (WGA) conjugated to FITC (Sigma) for 1 h at room temperature. Sections were washed in Tris-BSA (10 min x 3) and then mounted in anti-fade reagent Citifluor AF1 (Agar Scientific, UK). As negative controls for cytochemical labelling, WGA-FITC was replaced by Tris-BSA. Sections were imaged with a Zeiss LSM700 confocal microscope and images processed using ZEN 2011 software (Carl Zeiss) and Adobe Photoshop software.

For transmission electron microscopy, two-week-old *A. thaliana* Col-0 roots grown in half MS

medium with sucrose were dip-inoculated with *Ct*-GFP spores (2-5 x $10^5$ spores/ml) for 5 minutes and transferred to half-strength MS medium without sucrose after brief washing with DW. The infected roots were cryo-fixed by high pressure freezing at 3 dpi, 5 dpi and 7 dpi. Infected root segments were placed in 4.6 mm-diameter aluminium specimen carriers with 200 µm deep cavities (Leica Microsystems GmbH), mounted in 1-hexadecene, capped with a second specimen carrier (flat side towards the sample) and immediately frozen using a Leica EM HPM 100 high-pressure freezer (Leica Microsystems GmbH). Freeze-substitution in acetone containing 2 % (w/v) osmium tetroxide and 0.2 % (w/v) uranyl acetate was performed in a Leica EM AFS2 freeze substitution device (Leica Microsystems GmbH) according to Micali et al. (2011). After rinsing in acetone, samples were embedded in Agar low viscosity epoxy resin (Plano GmbH, Wetzlar, Germany) over a period of 6 days and polymerized in flat embedding molds at 60 °C for 24 h. Ultrathin sections (70 to 90 nm) were collected on copper slot grids as described by Moran and Rowley (1987). Sections were stained with 2 % uranyl acetate for 10 min followed by lead citrate for 15 min. Sections were examined with an Hitachi H-7650 TEM operating at 100 kV fitted with an AMT XR41-M digital camera (Advanced Microscopy Techniques, Danvers, USA). Images were processed using Adobe Photoshop software.

**Plant growth assay**

To quantify plant growth promotion mediated by *Ct* on *A. thaliana* plants grown in agar plates we used two different spore inoculation methods. In the first method, conidia and mycelium of *Ct* (1 x $10^6$ spores /ml and mycelium obtained after scratching surface of the fungal colony on Mathur's nutrient medium with 3 % agar (Freeman and Rodriguez 1992)) were mixed with molten agar (1 % w/v) and half-strength MS agar growth medium without sucrose in a 1:10 (v/v) ratio after the temperature of the medium had reached 30 °C. A total of 50 ml of the mixture was then poured into square Petri plates (15 cm x 15 cm). For the mock treatment, DW was mixed with the medium in a 1:10 (v/v) ratio. Seedlings of *A. thaliana* Col-0 were initially grown aseptically for seven days in half-strength MS agar medium with sucrose (1 %) and then transferred using sterile forceps to the (Pi)-sufficient or -deficient half-strength MS medium containing the fungal inoculum without sucrose (high Pi: 625 µM $KH_2PO_4$, low Pi: 50 µM $KH_2PO_4$). On each plate, five plants were incubated for 18 days at 21°C with a 10-hour photoperiod (80 µE/$m^2$s) in the Panasonic MLR-352 controlled environment chamber before root length and shoot fresh weight were determined. To minimize the light variations on plant growth the position of the square Petri plates within the chamber was frequently changed during

the incubation. In the second method, *Ct* or *Ci* conidia (~5 x $10^4$ spores/ml) were inoculated on surface-sterilized *A. thaliana* seeds. The *Ct* or *Ci*-inoculated seeds were then directly placed on half-strength low Pi MS medium without sucrose and incubated for at least 24 days using the same growth chamber conditions as described above. Heat-killed fungus was obtained by autoclaving at 121 °C for 15 min. The protocol for preparation of half-strength MS medium and plant growth conditions used in this study are described in Gruber et al. (2013) with one minor modification (pH=5.1). For plant growth promotion assays when plant-inaccessible hydroxyapatite provided the sole Pi source, we added 500 µM hydroxyapatite (Nacalai tesque) instead of 50 µM $KH_2PO_4$ at pH=5.7. We observed a clear phosphate starvation response phenotype in the mock-treated plants under these conditions. For plant growth promotion assays in vermiculite matrix (GS, NITTAI), *Ct* mycelium grown in Mathur's nutrient medium with 3 % agar (Freeman and Rodriguez 1992) was collected by scratching the plate surface with plastic micropipette tips. Surface mycelia collected from 10 agar plates were rinsed several times in DW. The washed mycelium was mixed with the vermiculite matrix in a 1:10 ratio (by weight) and subsequently 12-day-old *A. thaliana* plants grown in half-strength MS with 1 % sucrose were transferred to the vermiculite with *Ct*. The shoots were photographed 30 days post inoculation and the number of siliques was counted two months after inoculation. *ein2 pad4 sid2 dde2* quadruple mutant seeds were provided by Kenich Tsuda. *phr1, phr1 phl1, and phf1* mutant seeds were provided by Javier Paz-Ares. *myb34 myb51 myb122* triple seeds were provided by Tamara Gigolashvili. *phl1* mutant seeds (SAIL_731_B09) and transgenic seeds expressing PIP2A fused with mCherry were provided by NASC. *C. incanum* strain (MAFF 238706) was provided by NIAS genebank in Japan.

**$^{33}$P translocation experiment**

Surface-sterilized *A. thaliana* Col-0 seeds were placed on MS plates, supplemented with 0.8 % sucrose, and germinated in a controlled-environment chamber for approximately seven days. Fungi were cultivated on PDA (Difco) agar plates at room temperature in the dark. For $^{33}$P translocation experiments, square Petri plates (root/hyphal compartment, RHC; 12 x 12 cm) were used. Inside these plates, a small, circular Petri plate (hyphal compartment, HC; 3.8 cm in diameter) was placed at the bottom (see Figures 3D, and S4E) and both plates were filled with MS-medium to the brim of the small plate. In this way, the RHC was separated from the HC by the plastic wall of the small plate. Two cm of agar was removed from the top of the large plate

to provide space for growing shoots. Two PDA agar blocks with or without fungal material were added to the HC and the two-compartment system was incubated in a controlled-environment chamber for 7 days (10 h light, 14 h darkness, 22 °C, 60% relative humidity, 100 µE/$m^2$s white light). During this incubation period the fungus grew from the HC to the RHC, bridging the wall of the small plate with its hyphae. At this stage two one-week-old Col-0 seedlings were added per plate and cultivated vertically in the phytochamber for another week. When fungal hyphae reached the plant roots, 270 kBq of carrier-free $^{33}$P-labelled $H_3PO_4$ (Hartmann Analytik GmbH, Braunschweig, Germany) were added to the HC. Plates were placed horizontally in the controlled-environment chamber for 3 days and subsequently moved to a 60° tilted position. Plants and fungi were co-cultivated for another 14 days in the presence of $^{33}$P. At harvest, plates were scanned using the WinRHIZO scanner and software (Regent Instruments, Inc., Canada) and shoots were harvested and dried to constant weight at 65 °C. After dry weight determination, each plant was digested with 500 µl $HNO_3$ at 100°C for 20 min. After adding $H_2O_2$ (250 µl), the mixture was heated again to 100°C. Five hundred µl of this solution were mixed with 4.5 ml of scintillation cocktail (Rotiszint eco plus, Roth, Karlsruhe, Germany) and used for detection of $^{33}$P signals with a scintillation counter (Beckman Coulter LS 6500, Krefeld, Germany).

**RNA-seq experiments**

Mock treated or colonized roots were collected at 6, 10, 16, and 24 dpi (*C. tofieldiae* 0861 (*Ct*)-Arabidopsis interaction). Plants were grown in vitro on a defined agarose medium using either high [625 µM] or low [50 µM] phosphate concentrations. RNA-purification with the NucleoSpin RNA Plant kit (Macherey-Nagel) was performed according to the manufacturer's protocol. RNA-seq libraries were prepared from an input of 1 µg total RNA using the Illumina TruSeq™ Stranded RNA Sample Preparation Kit. Further details are provided in Hacquard et al. (submitted). Illumina sequencing produced 20 to 45 million paired-end fragments (read length 100 bp) per sample. Reads were mapped to the annotated genomes of *Ct* (Hacquard et al., submitted) and *A. thaliana* (TAIR10) using the splice aware read aligner Tophat2 (Kim et al., 2013) as described previously (Hacquard et al., submitted). The mapped RNA-seq reads were subsequently transformed into a fragment count per gene per sample using the htseq-count script (s=reverse, t=exon) in the package HTSeq (Anders et al., 2015). Statistical analyses of plant and fungal gene expression were performed using the R package 'limma' as described in Hacquard et al. (submitted). Resulting *p*-values were adjusted for false discoveries (FDR) due to

multiple hypotheses testing via the Benjamini-Hochberg procedure. To identify genes with significant expression differences, a cut-off of FDR < 0.05 and |log2FC| ≥ 1 was applied. Heatmaps of gene expression profiles were generated with the Genesis expression analysis package (Sturn et al., 2002). The RNA-seq data used in this study are available under the GEO series accession number GSE70094 from the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database. The Gene Ontology (GO) analysis described in Figure S4 was conducted by agriGO (Zhou et al., 2010).

**Quantitative real-time PCR**

Three biological replicates were obtained for each sample. cDNA was synthesized from 500 ng total RNA using the PrimeScript RT Master Mix (Takara) in a volume of 10 µL. Five µL of cDNA (10 ng/µL) was amplified in SYBR® Premix Ex Taq™ II with 1.6 µM primers using the Thermal Cycle Dice Real Time System (TaKaRa). Primers used in this study are listed in Table S1.

**Elemental analysis by ICP-MS**

Conidia and mycelium of *Ct* (1 x $10^6$ conidia per ml) were mixed with molten half-strength MS agar growth medium in a 1:10 (v/v) ratio and 50 ml of the mixture was poured into square Petri plates (15 cm x 15 cm). For mock treatments, DW was mixed with the medium in a 1:10 (v/v) ratio. Surface-sterilized seedlings of *A. thaliana* Col-0 were grown aseptically for seven days in half-strength MS agar medium with sucrose and then transferred using sterile forceps to Pi-limiting medium without sucrose. Roots and shoots of *A. thaliana* plants were collected after 18-day incubation and digested individually in 500 µl of concentrated (66%) nitric acid by heating for 2 h at 100 °C. After digestion, the acid concentration was reduced by 1:10 dilution with water. Determination of phosphorus was performed with an Agilent 7700 ICP-MS (Agilent) following the manufacturer's instructions.

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