Exogenous dopamine and overexpression of the dopamine synthase gene *MdTYDC* alleviated apple replant disease

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Abstract

Apple replant disease (ARD) is a soil-borne disease that leads to economic losses due to reduced plant growth and diminished fruit yields. Dopamine is involved in interactions between plants and pathogens. However, it remains unclear whether dopamine can directly stimulate defense responses to ARD. In this study, an exogenous dopamine treatment and dopamine synthetase *MdTYDC* (tyrosine decarboxylase) transgenic plants were used to verify the role of dopamine in treating ARD. First, two-year-old trees of *Malus domestica* cv. Fuji apple, grafted onto rootstock M.26, were grown in replant soils. The addition of dopamine (100 μ*M*) to the soil promoted seedling growth and changed the accumulation of mineral elements in plants in replant soils. Such supplementation improved the activity of invertase, urease, proteinase, and phosphatase under replant conditions. Sequencing analysis of 16S rDNA and ITS rDNA revealed that dopamine had a slight influence on bacterial diversity, but had an obvious effect on fungal diversity in replant soils. The application of dopamine to replant soil changed the composition of bacterial and fungal communities. Second, overexpression of *MdTYDC* in apple plants alleviated the effects of ARD. *MdTYDC* transgenic lines exhibited mitigated ARD through inhibited degradation of photosynthetic pigment, maintaining the stability of photosystem I and II, and improving the antioxidant system. Furthermore, overexpression of *MdTYDC* improved arbuscular mycorrhizal fungi colonization by improving the accumulation of soluble sugars under replant conditions. Together these results demonstrated that dopamine enhances the tolerance of apples to ARD.

Keywords: *MdTYDC*, tyrosine decarboxylase, soil enzyme activity, soil microbial composition, photosynthesis, arbuscular mycorrhizal fungi.

1. Introduction

Apple is one of the most beneficial fruits for human health. Globally, China is the largest region of apple production. Trees in most of China's prominent areas of cultivation have passed their period of maximum yield and are entering the senescence phase, with approximately 70% of the orchards more than 20 years old (Sun et al. 2014). Land for fruit crops is scarce in China, and the same fields are commonly used for repeated production—conditions in which apple replant disease (ARD) has become widespread. ARD is a soil-borne disease that typically causes reduced plant growth, yellowing of foliage, shortened internodes, and root systems that are small, discolored, and inhibited in their development, all of which lead to a shortened span of productivity and reduced yields (Mazzola and Manici 2012).

Causes of ARD have been attributed to abiotic factors, including extremes in pH levels, heavy-metal contamination, cold or drought stress, and poor soil nutrition or structure. Most experimental evidence suggests that the disease also may be caused by biotic factors (Mazzola and Manici 2012). Soil microorganisms are considered as an important component of soil function because they are involved in mineral nutrient release, plant disease resistance, and the spread of diseases (Franke-Whittle et al. 2015). Assumed causal pathogens include bacteria, nematodes, oomycetes, and soil-borne fungi, all of which are found in various combinations in soils (Kviklys et al. 2016). The etiology of ARD is unclear due to its complexity, but the most endorsed hypothesis hings on changes in soil microbial communities (Mazzola and Manici, 2012). Herbaceous plants and trees also can affect microbial community composition in their rhizospheres, largely due to the quality and quantity of organic substances released by roots (which are carbon and energy sources for microorganisms) (Mazzola and Manici 2012; Del-Saz et al. 2017). Even small amounts of biocidal compounds secreted by roots may significantly affect rhizosphere microbial community composition (Rumberger and Marschner 2003; Weiss et al. 2017). The application of melatonin to replant soil increases soil enzyme activity and soil quality while altering the composition of bacterial and fungal communities (Li et al., 2018). Also, apple roots normally colonized by arbuscular mycorrhizal fungi (AMF) have improved rootstock growth when placed in soil conducive to specific ARDs (Ridgway et al. 2008).

Dopamine, a member of the catecholamine group, was first discovered in the mammalian brain. It is common in animals and plays key roles in the central nervous system and normal body physiological

activities (Guo and Dong 2005; Qian et al. 2013; Huang et al. 2016). Dopamine also has been identified as an antioxidant in plants, with similar efficacy to ascorbic acid and gallocatechin gallate (Kulma and Szopa 2007). We previously have shown that dopamine alleviates the impacts of nutrient deficiency-induced or salt-induced stress in apple (Li et al. 2015; Liang et al. 2017). Other studies have demonstrated that this molecule is involved in interactions between plants and pathogens. Synthesis of dopamine can be activated by virus infections (Swiedrych et al. 2004b). Catecholamines improve the resistance of plants to pathogens and participate in nitrogen detoxification (Kulma and Szopa 2007). Previous studies have shown that the resistance of plants to pathogen infection is positively correlated with catecholamine content (Swiedrych et al. 2004b). In plants, the precursors of dopamine, tyramine and L-dopa, are produced via either tyrosine hydroxylase (TH) or TYDC (Kulma and Szopa 2007; Rezaei et al. 2016). TYDC acts as a key enzyme in dopamine synthesis, and its catalytic products can inhibit fungal mycelium growth (Facchini and Luca 1995; Negrel and Javelle 2001; Deloache et al. 2015). For example, the expression of *TYDC* is induced by a pathogen-derived elicitor in cultured opium poppy (*Papaver somniferum* L.) (Park et al. 1999). Transgenic *TYDC* potato plants have greater resistance to potato virus Y and *Erwinia carotovora* infection (Swiedrych et al. 2004a). Such results suggest that dopamine is active in the plant response to infection. Yet, it remains unclear whether dopamine can directly stimulate defense responses to ARD.

We studied if exogenous dopamine supplementation and overexpression of *MdTYDC* (LOC103448183) could alleviate the effects of ARD. We explored the effects of exogenous dopamine on apple plant growth, soil enzyme activity, and soil microbial composition, and effects of overexpression of *MdTYDC* on photosynthesis, chlorophyll fluorescence parameters, and root antioxidant enzymes.

2. Materials and methods

2.1 Exogenous dopamine-treated apple plants

2.1.1 Plant materials and growing conditions

All experiments were conducted at Northwest A&F University, Yangling (34°20´N, 108°24´E), Shaanxi, China. The replant soil and control soil (referred to hereafter as "control") were collected from a thirty-year-old apple orchard and a vegetable cultivation site near the orchard, respectively. After removing leaves and other loose materials from the surface, soils were collected from multiple randomly selected sites at a depth of 20–30 cm. Before use, soils were mixed and sieved through a 2 mm mesh. This experiment was conducted with two-year-old apple trees (*M. domestica* cv. Fuji) grafted onto rootstock M.26. One hundred apple trees planted in pots (38 cm × 23 cm) filled with control soil and one hundred apple trees were grown in the same-sized pots filled with "replant" soil. 100 μ*M* dopamine was supplemented every 20 days from April to September 2016. Four experimental groups were established: 1) CK, plants grown with control soil; 2) ACK, plants grown in replant soil; 3) DA, dopamine control, control soil supplemented with 100 μ*M* dopamine; and 4) ADA, a combination of replant soil plus 100 μ*M* dopamine treatment. The physiological properties for each group were evaluated at the end of the experiment.

2.1.2 Growth and mineral element measurements

At the end of the trial period, 15 plants were collected from each treatment. After an image of the leaf samples was captured with a scanner, their areas were measured by Image J software. Dry weights of root, stem, and leaf parts were determined after drying the tissues at 80°C for at least 72 h to a constant weight. Contents of nitrogen (N), phosphorus (P), and potassium (K) were measured according to Li et al. (2015).

2.1.3 Measurements of dopamine contents

The content of dopamine was determined according to our previous study (Gao et al. 2020).

2.1.4 Activity of soil enzymes

After sieving with a 2 mm sieve, the air-dried soil was stored at 4 °C until soil enzyme activity was measured. Activities of invertase, urease, and neutral phosphatase in soils were examined according to previous studies (Guan 1986). In brief, the activity of invertase was detected by the 3,5-dinitro salicylic acid method using sucrose as substrate, and the activity is expressed as the weight of glucose released per gram of soil per 24 h of incubation. Urease activity was measured by indophenol colorimetry using

urea as substrate. Briefly, 5 g dry soil was incubated with 10% urea as substrate and phosphate buffer (0.2 M, pH 8.0, to bring the reaction mixture to pH 7.0 or slightly above 7.0) at 37°C for 24 h. The activity is expressed as the weight of ammonia per gram of soil per 24 h. Neutral phosphatase enzyme activity was determined by disodium phenyl phosphate colorimetry using p-nitrophenyl phosphate as substrate. After incubation at 37°C for 24 h, the activity was expressed as nmol phenol g^{-1} soil 24 h^{-1}. The activity of proteinase was determined by ninhydrin colorimetry according to Ladd and Butler (1972). Proteinase activity was expressed as the weight of amine N produced per gram of soil per 24 h of incubation.

2.1.5 Soil DNA extractions and Illumina sequencing

At the end of the trial period, bulk soil and plants were gently removed and sent to the laboratory. Loosely adhering soil was removed from the root surface and rhizosphere soil was obtained, then was sieved through a 2 mm mesh. To ensure the representativeness of samples, three biological replicates were used for each treatment group, and each replicate comprised samples from five mixed pots. Soil samples were stored at -80°C for DNA extraction.

DNA was extracted from 1 g samples with a Fast DNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). The diversity and composition of bacterial communities in each soil sample were determined accord to Caporaso et al. (2010). PCR with the 341F (5′- CCTAYGGGRBGCASCAG -3′) and 806R (5′- GGACTACNNGGGTATCTAAT -3′) primer sets amplified the V3-V4 region of the 16S rDNA gene. PCR was performed in 50 μL mixture containing 5 μL 10 × KOD Buffer, 5 μL 2.5 mM dNTPs, 1.5 μL 5 μ*M* primer, 1 μL KOD Polymerase, and 100 ng template DNA. PCR was performed at 95°C for 2 min, followed by 27 cycles of 98°C for 30 s, annealing at 62°C for 30 s, 68°C for 30 s, and a final extension at 68°C for 10 min. The diversity and composition of fungal communities were determined by amplifying internal transcribed spacer 2 (ITS2) genes. PCR with fungal-specific forward primer F (5′-GCATCGATGAAGAACGCAGC-3′) and reverse primer R (5′-ATATGTAGGATGAAGAACGYAGYRAA-3′) sets amplified the ITS2 region of the eukaryotic ribosomal RNA gene. PCR was performed in 50 μL mixture contained 5 μL 10 × KOD Buffer, 5 μL 2.5 mM dNTPs, 1.5 μL 5 μ*M* primer, 1 μL KOD Polymerase, and 100 ng template DNA. PCR was performed at 95°C for 2 min, followed by 27 cycles at 98°C for 10 s, 62°C for 30 s, and 68°C for 30 s and a final extension at 68°C for 10 min. After, the amplicons were pooled, purified, and quantified by

Nanodrop procedures using the Illumina Hiseq 2500 PE250 platform (Illumina, San Diego, CA, USA). The high-quality sequences were clustered into operational taxonomic units (OTUs) defined based on ≥ 97% similarity using the UPARSE pipeline (v9.2.64_i86linux32; Edgar 2013). The Ribosomal Database Project classifier against the Greengenes Database (version 20101006; DeSantis et al. 2006; Wang et al. 2007) was used for the taxonomic assignment of bacteria. The UNITE reference sequence database (http://unite.ut.ee) was used for the taxonomic assignment of fungi.

2.2 MdTYDC transgenic apple plants

2.2.1 Plant materials and growing conditions

M. domestica cv. 'Roya Gala' apple *in vitro* shoot cultures were used for genetic transformation (unpublished data). They were grown on MS subculture media containing 0.2 mg L^{-1} IAA and 0.3 mg L^{-1} 6-BA under a 14 h photoperiod with a light intensity of 60 μmol m^{-2} s^{-1}. Then, transgenic and WT plantlet main shoots were cut into 1.5 cm segments and were rooted in an MS agar medium containing 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} IBA. This experiment was conducted with two-year-old, pot-cultured WT and transgenic apple trees. Replant treatments were the same as in the above-outlined experiment. After 3 months of treatment, growth parameters were recorded and various tissues types sampled and frozen in liquid nitrogen.

2.2.2 Photosynthesis and photosynthetic pigment content

P_n (Net photosynthesis), g_s (stomatal conductance), and C_i (intercellular CO_2) concentration were determined using a portable system (Li-6400; LICOR, Huntington Beach, CA, USA) set at 1000 μmol m^{-2} s^{-1} quantum flux and a 400 μmol mol^{-1} CO_2 concentration. Leaf chlorophyll was extracted with 80% acetone and determined spectrophotometrically according to Arnon (1949).

2.2.3 Chlorophyll fluorescence parameters

Removed apple leaves were adapted to darkness for 20 min, and chlorophyll fluorescence was measured using a FlourCm FC800 (Photon Systems Instruments, Drasov, Czech Republic) containing a fast fluorescence induction (OJIP) and a CCD camera.

Chlorophyll fluorescence parameters were measured at 9:00–11:00 on a sunny day. Apple leaves were adapted to darkness for 20 min by attaching light-exclusion clips to the same region of each leaf.

Then chlorophyll fluorescence parameters (F_V/F_M), Y (I), Y (II), ETR (I), and ETR (II)) were measured using a PAM-2100 fluorimeter (Walz GmbH, Effeltrich, Germany).

2.2.4 H_2O_2, O_2^-, and antioxidant enzymes

H_2O_2 was extracted with 1% (w/v) polyvinylpolypyrrolidone and measured according to Patterson et al. (1984). O_2^- was measured according to Elstner and Heupel (1976). The ground sample (0.1 g) was suspended in 1 ml 10 m*M* phosphate buffer and centrifuged at 10,000g for 3 min. SOD, POD, and CAT activities were determined using colorimetric assay kits (Keming Bioengineer Company, China).

2.2.5 Determination of AM colonization

Fresh root samples were washed and chopped into 1.0 cm long segments, then rinsed and cleared with 10% KOH, and finally, stained with 0.05% trypan blue in lactic acid. Afterward, the root segments (50 segments for each plant, 5 plants for each treatment) were examined with an SFC-18 Motic microscope. The AM colonization was calculated according to Biermann and Linderman (1981).

2.2.6 Measurements of soluble sugars

Frozen root samples (0.1 g) were ground, then the contents of sucrose, glucose, and fructose were determined using colorimetric assay kits (Keming Bioengineer Company, China).

2.2.7 Real-time PCR (RT-PCR) analysis

Total RNA was extracted accord to Chang et al. (1993). All primers (see Table S1 available as Supplementary Data at Tree Physiology Online) were designed by P_{RIMER} P_{REMIER} 6 software (Biosoft International, Palo Alto, CA). Poly $(A)^+$ RNA was purified using poly $(A)^+$ Ttract® mRNA Isolation Systems III kit (Promega, Madison, WI). RT-PCR was performed on an iQ5.0 instrument (Bio-Rad, USA) using SYBR Green qPCR kits (TaKaRa). In this study, the *Malus* elongation factor 1 alpha gene (*EF-1a*; DQ341381) was used to standardize the cDNA samples for different genes. To verify the specificity and amplification efficiency of primers, we monitored the identity and specificity of these RT-PCR products by conducting a melting-curve analysis.

2.3 Statistical analysis

Based on the abundance information of OTUs, a principal component analysis was carried out using R (Jombart 2008) and α-diversity index (Shannon, Chao, ACE) calculated with Mothur software. Physiological data were determined using a one-way analysis of variance (ANOVA), followed by Tukey's tests. P-values < 0.05 indicated significant differences. Data analyses were performed using SPSS 17.0 (IBM, Chicago, Illinois, USA), and the figures were developed in SigmaPlot 10.0 (Systat Software, California, USA).

3. Results

3.1 Exogenous dopamine-treated apple

3.1.1 Apple growth

Exogenous dopamine application significantly increased the content of dopamine in plant leaves under both control and replant conditions (P = 0.006 and P = 0.005, respectively; see Figure S1 available as Supplementary Data at Tree Physiology Online). Under replant conditions, the growth of apple trees was significantly affected (Table 1; see Figure S2 available as Supplementary Data at Tree Physiology Online). Exogenous application of 100 μM dopamine alleviated this response. Compared with the CK treatment, reductions in ACK versus ADA plants were: 47.51% vs 29.03%, 59.11% vs 37.29%, 22.60% vs 9.47%, 32.25 vs 18.64%, and 35.96% vs 18.18% for root dry weight, stem dry weight, height, stem diameter, and leaf area respectively.

3.1.2 Levels of N, P, and K

In ARD soils, there were no significant differences in N concentrations in leaves and stems between ACK and ADA treatments (P > 0.05; Figure 1A and 1B). N levels in roots were significantly higher in the ADA group than in ACK plants (P = 0.015; Figure 1C). ARD led to significant increases in concentrations P in leaves (Figure 1D). Compared with ACK plants, P levels in leaves were significantly higher in the ADA plants (P = 0.028). There were no significant differences in P concentrations in stems and roots among all four treatments (P > 0.05; Figure 1E and 1F). In ARD soils, exogenous dopamine had no significant effect on K levels in leaves (P > 0.05), but dopamine-treated

plants had higher K levels in stems and roots (P = 0.023 and P = 0.011, respectively; Figure 1G, H, and I).

3.1.3 Soil enzyme activity

In ARD soils, the activity of soil invertase was significantly higher in the ADA group than in the ACK group (P < 0.001; Figure 2A). Urease activity significantly decreased in the ACK group compared with the CK group and was significantly elevated in the ADA group compared with the DA group (P = 0.014 and P = 0.029, respectively; Figure 2B). ARD significantly reduced the activity of proteinase in soils. However, the ADA group had higher proteinase activity than the ACK group (P < 0.001; Figure 2C). In ARD soils, the activity of phosphatase was significantly higher in the ADA group than in the ACK group (P = 0.003; Figure 2D). Therefore, the activity of all four enzymes in replant soil was significantly increased by the addition of dopamine.

3.1.4 Richness and diversity of rhizosphere bacteria and fungi

The richness and diversity of bacteria and fungi were observed based on Chao, Ace, and Shannon indexes in different groups (Table 2). Operational taxonomic units (OTUs) were identified based on the conventional criterion of 97% similarity. Microbial richness was characterized by the Chao and Ace indexes. There was no significant difference in richness and diversity of bacteria among all samples (P > 0.05). However, compared with the ACK group, the ADA group displayed a significantly higher diversity of fungi (P = 0.003). Principal component analysis (PCA) showed that the application of dopamine had no significant influence on bacterial diversity, but a significant effect on fungal diversity (see Figure S3 available as Supplementary Data at Tree Physiology Online).

3.1.5 Taxonomic coverage for 16s rDNA

Actinobacteria, Gemmatimonadetes, Acidobacteria, Planctomycetes, and Proteobacteria were the five most dominant phyla in all samples, accounting for >70% of the reads (Figure 3A; see Table S2 available as Supplementary Data at Tree Physiology Online). Importantly, in CK and ADA treatments, there were more unclassified species, consistent with higher diversity indices. Compared with other samples, ACK had a significantly higher percentage of Acidobacteria (CK:

9.73%, ACK: 13.69%, DA:11.23%, ADA: 13.32%) and Nitrospirae (CK: 0.68%, ACK: 1.49%, DA: 1.01%, ADA: 1.23%), but a lower percentage of Chloroflexi (CK: 2.99%, ACK: 2.85%, DA: 3.12%, ADA: 2.97%) and *OD1* species (CK: 2.90%, ACK: 1.76%, DA: 2.65%, ADA: 1.86%). At the class level, compared with other samples, ADA had a significantly higher percentage of Phycisphaerae and Planctomycetia, but a lower percentage of Actinobacteria, Cytophagia, and Gammaproteobacteria (Figure 3B; see Table S2 available as Supplementary Data at Tree Physiology Online).

At the genus level, exogenous dopamine significantly increased amounts of *Lactobacillus* (32.29-fold) and *Frankia* (5.30-fold), while significantly reducing levels of *Azoarcus* (0.25-fold), *Sphingobium* (0.19-fold), and *Azorhizophilus* (0.04-fold) in control soils (Figure 4; see Table S3 available as Supplementary Data at Tree Physiology Online). Compared with ACK, ADA had significantly more *Acetobacter* (23.74-fold) and *Lactobacillus* (27.17-fold), but fewer *Azoarcus* (0.20-fold), *Geobacter* (0.20-fold), and *Arthrobacter* (0.19-fold).

3.1.6 Taxonomic coverage for ITS rDNA

All sequences were classified into five phyla. Ascomycota, Basidiomycota, and Ciliophora were the most dominant in all samples, accounting for >95% of the reads (Figure 5A; see Table S4 available as Supplementary Data at Tree Physiology Online). Compared with other samples, ACK had a significantly higher percentage of Ascomycota, and lower percentages of Ciliophora, Basidiomycota, and Glomeromycota. At the class level, compared with other samples, ACK had a significantly higher percentage of Sordariomycetes (2.18- to 3.22-fold) and lower percentages of Orbiliomycetes (CK: 2.77%, ACK: 0.08%, DA: 1.17%, ADA: 0.14%), Agaricomycetes (CK: 13.71%, ACK: 9.44%, DA: 10.86%, ADA: 11.63%), and Glomeromycetes (CK: 1.74%, ACK: 0.62%, DA: 1.72%, ADA: 3.13%) (Figure 5B; see Table S4 available as Supplementary Data at Tree Physiology Online). Compared with the ACK group, ADA led to significantly higher percentages of Pezizomycetes (7.78-fold), Tremellomycetes (3.00-fold), and Glomeromycetes (4.36-fold).

At the genus level, compared with CK, DA showed significantly more *Amphobotrys* (486-fold), *Volutella* (172-fold), and *Stachybotrys* (416-fold), but fewer *Mycoarthris* (0.001-fold), *Clonostachys* (0.001-fold), and *Scleropezicula* (0.003-fold) (Figure 6; see Table S5 available as Supplementary Data at Tree Physiology Online). In replant soils, exogenous dopamine significantly increased amounts of *Amphobotrys* (486-fold), *Volutella* (172-fold), and *Stachybotrys* (416-fold), while significantly reducing amounts of *Mycoarthris* (0.001-fold), *Clonostachys* (0.001-fold), and *Scleropezicula* (0.003-fold). *Slopeiomyces and Pleurotheciella* were only found in replant soils. *Exophiala*, *Lectera*, and *Microsporum* were found in all soil groups except ADA.

3.2 MdTYDC transgenic apple plants

3.2.1 MdTYDC overexpression alleviated apple replant disease

TYDC acts as a key enzyme in dopamine synthesis, and three transgenic lines (OE-2, OE-3, and OE-5) of *MdTYDC*-OE apple were obtained for further examination of the function of dopamine in replant soil. Transgenic lines OE-2, OE-3, and OE-5 significantly increased dopamine contents in apple leaves (Figure 7A). Under control conditions, the expression of *MdTYDC* of leaves and roots in transgenic lines was up-regulated 2-4 times relative to that in WT (Figure 7B and 7C). In leaves, there was no significant difference in the expression of *MdTYDC* between control soils and replant soils in all lines. However, in roots, ARD significantly increased the expression of *MdTYDC* in WT and transgenic lines. This indicates that *MdTYDC* in roots may respond to ARD. In replant soil, lines OE-2, OE-3, and OE-5 exhibited higher shoot and root dry weight. Lines OE-3 and OE-5 had significantly higher plant height than WT in replant soil (Table 3 and Figure 7D). These results suggested that *MdTYDC* plays a positive role in plant response to ARD.

In replant soil, contents of chlorophyll a (Chl a) and total chlorophyll (Chl t) decreased in WT and all OE lines, and all OE lines had higher Chl a and Chl t contents than WT (Figure 8A and 8D). OE-3 and OE-5 lines had higher (chlorophyll b) Chl b and carotenoid (Car) contents than WT (Figure 7B and 7C). In response to ARD, P_n, g_s, and Ci decreased in WT (Figure 8E-G). In ARD soils, lines OE-2, OE-3, and OE-5 had increased values of P_n and Ci. Lines OE-2 and OE-3 had higher g_s than WT. This

suggested that *MdTYDC* limited the decrease in photosynthetic pigment content and photosynthetic capacity caused by ARD.

3.2.2 Chlorophyll fluorescence parameters

Chlorophyll fluorescence parameters are closely related to various responses of photosynthesis. In ARD soils, F_V/F_M decreased in WT, whereas in OE lines there was no significant change (Figure 9A and 9B). Under control conditions, Y (I), ETR (I), and ETR (II) did not differ significantly between OE lines and WT (Figure 9C, 8E, and 8F). OE lines exhibited higher ETR (I) activity than WT. In ARD soils, OE-2 and OE-5 had higher activity of Y (I) than WT. OE-2 had higher Y(II) activity than other lines under control conditions, whereas OE-2 and OE-3 lines had higher Y(II) activity than WT in ARD soils (Figure 9D). In ARD soils, only the OE-3 line had higher activity in ETR (II) than WT (Figure 9F). These results suggested that *MdTYDC* plays a positive role in the activity of PSI and PSII under pathogenic pressure.

3.2.3 H_2O_2, O_2^-, antioxidant enzymes, and expression of antioxidase-related genes in the ascorbate–glutathione (AsA-GSH) cycle in roots

In control soil, there were no differences in the contents of H_2O_2 and O_2^-, nor the activities of SOD, POD, and CAT, between WT and *MdTYDC* transgenic lines (Figure 10). All OE lines exhibited lower H_2O_2 contents, and OE-2 and OE-5 exhibited lower O_2^- contents than WT in ARD soils (Figure 10A and 10B). The activities of SOD in OE-2 and OE-5, and the activities of POD in OE-2 and OE-3, were significantly higher than for WT (Figure 10C and 10D). In ARD soils, the activities of CAT in all OE lines were higher than for WT (Figure 10E). This suggested that *MdTYDC* overexpression detoxified H_2O_2 and O_2^- through improved the activities of SOD, POD, and CAT. Because the AsA-GSH cycle also plays an important role in eliminating H_2O_2 and O_2^-, the expression of antioxidase-related genes in the AsA-GSH cycle in roots was determined (Figure 10F). In ARD soils, the expression of *MdDHAR1*, *MdcAPX*, *MdcGR*, and *MdMDHAR* in all OE lines was higher than for WT.

3.2.4 AMF colonization and soluble sugar content in roots

In our previous research, exogenous dopamine promoted mycorrhizal symbiosis (unpublished data). Therefore, we also examined AMF colonization in WT and *MdTYDC* transgenic plants in ARD soils (Figure 11A). In control and ARD soils, OE-3 and OE-5 displayed higher AMF colonization than WT.

Soluble sugar content in roots was also determined. There was no significant difference in the content of fructose between WT and *MdTYDC* transgenic lines in control or ARD soils (Figure 11D). However, in ARD soils, the content of sucrose and glucose in OE-3 and OE-5 was higher than that of WT (Figure 11B and 11C).

4. Discussion

4.1 Exogenous dopamine-mitigated ARD through regulated element accumulation and enhanced soil enzyme activity

In this study, ARD significantly inhibited plant growth development, and exogenous dopamine alleviated these inhibitory effects. Minerals in plant roots taken up from soil are essential for plant growth; the reason that dopamine promoted apple tree growth in replant soil is that it helped plants regulate the accumulation of elements. We found that the concentrations of root N, leaf P, and both root and stem K increased in dopamine-treated trees in replant soil, possibly due to dopamine regulation of the activity of soil enzymes.

Soil enzymes play an important role in maintaining soil physicochemical properties and in nutrient cycling (Das and Varma 2011). Invertase is involved in the hydrolysis of sucrose into glucose and fructose, and in breaking down cellulose. In this study, the activities of invertase and cellulase were lower in replant soil than those in control soil. However, those activities were significantly enhanced in response to dopamine supplementation. The long-term productivity of a soil can be reflected by its invertase activity (Mikhailouskaya and Bogdevitch 2009). This enzyme decomposes complex organic compounds into subunits absorbed by microorganisms, resulting in delayed changes in microbiological parameters, and higher microbial biomass turnover may lead to greater invertase activity (Kandeler et al. 1999). Urease acts as an important index of soil health and is involved in the hydrolysis of urea into ammonia and carbamate. In the present study, the activity of urease was higher in control than replant soil. However, under replant conditions, the application of dopamine leads the root exudates to support a different functional microbial community, which is the reason for the obvious increase in mineralization and nutrient utilization. Phosphatase also plays an important role in phosphorus mineralization, and proteinase is important in the carbon cycle (Gianfreda et al. 2005; Li et al. 2011). Our results indicated that the levels of both enzymes were higher in the ADA soil group than in the ACK samples, a result consistent with that from previous studies. The effects of dopamine on the interaction

between the rhizosphere environment and microorganisms, and how this interaction affects soil enzyme activity, warrants further study.

4.2 Effects of exogenous dopamine on soil microbial communities

Soil microbial communities play an important role in organic matter decomposition, nutrient cycling and energy flow, which are related to the stability and function of soil systems (Franke-Whittle et al. 2015). We examined the effects of dopamine on rhizosphere bacteria and fungi populations, and found there were significant differences in microbial community composition between control and replant soil, which was further amplified when dopamine was applied. Although the overall composition of bacteria between ACK and ADA was similar, some differences in phylum or other groups were apparent. Actinobacteria, Gemmatimonadetes, Acidobacteria, Planctomycetes, and Proteobacteria were predominant in all samples; however, the replant soil that was not treated with dopamine presented a unique combination of phyla, including a higher proportion of Acidobacteria and Nitrospirae, and a lower percentage of Chloroflexi and OD1. This may be explained by the "rhizosphere effect," that is, some root tissue may remain in the replant sites. Control soil treated with dopamine had significantly higher percentages of Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, OD1, and Proteobacteria, and lower percentages of Planctomycetes and Verrucomicrobia. The phylum Acidobacteria often is dominant in soils (Zhang et al. 2013) and this was also supported by our findings. However, bacteria within that phylum occur more frequently in control plant rhizospheres than in the diseased plant rhizospheres (Yin et al. 2013), which is not consistent with our study. Although the number of sequences analyzed was limited, these current results provide valuable insight into the poorly understood microbial ecosystem associated with the effects of dopamine on ARD. Members of Bacteroidetes have been used as a biological index of agricultural soil usage. Proportions of Bacteroidetes vary among different soil environments, but they have rarely been explored for plant growth—probably because they are only a minor component of most soil samples. This is another potential area for future study.

We found that dopamine had an obvious influence on fungi community composition and diversity at the phylum and class levels. In addition to the dominance of Ascomycota and Basidiomycota in all samples, a large number of Ciliophora were detected. However, only a small proportion of fungal diversity has been analyzed (Hibbett et al. 2011), so additional research should focus on the importance

of soil fungi communities when targeting improved soil health. In our research, replant soil without dopamine had a significantly higher percentage of Ascomycota and lower percentages of Ciliophora, Basidiomycota, and Glomeromycota. We found it noteworthy that in replant soil, the percentage of Glomeromycota was higher in the presence of dopamine than when the soil had no such treatment. AMF belongs to the phylum Glomeromycota, a group that plays a critical role in plant nutrition and soil fertility (Harley and Smith 2008). Mycorrhizae improve host plant defense against many soil-borne fungal pathogens (Harley and Smith 2008). They have been shown to increase the resistance of tomato (*Lycopersicon esculentum* L.) to soil-borne diseases caused by *Phytophthora nicotianae* (Cordier and Gianinazzi-Pearson 1996), as well as to foliar diseases caused by the necrotrophic fungus *Alternaria solani* (Fritz et al. 2006). The roles of pathogens in decreasing soil fertility are varied, and here we suggest that the low relative abundance of Glomeromycota in non-dopamine replant soil might be part of the explanation. For other fungi phyla, even less is known.

Dopamine significantly increased proportions of Pezizomycetes, Tremellomycetes, and Glomeromycetes in replant soil. Three genera within Pezizomycetes are considered to be plant pathogens (*Rhizina*, *Caloscypha*, and *Strumella*), and most species in that class are believed to show saprobic or mycorrhizal properties (Tedersoo and Smith 2013). Many fungi in Glomeromycetes establish AM relationships with various plant species (Spain et al. 2006). Tremellomycetes, a class within Agaricomycotina, dimorphic taxa, encompasses yeasts, and species that form hyphae and/or complex fruiting bodies (Liu et al. 2015). Little has been published about their pathogenicity. We also observed that the addition of dopamine to replant soil significantly decreased the proportion of Sordariomycetes. Members of that class are ubiquitous, functioning in almost all ecosystems, and associated with arthropods, mammals, pathogens, and endophytes of plants, mycoparasites, and saprobes. Saprobic Sordariomycetes function in the decomposition and nutrient-cycling of plant litter (Zhang et al. 2006). This phenomenon can be explained by the "rhizosphere effect."

It is still not clear how dopamine regulates the overall composition of soil microbial communities. Dopamine participates in the growth and development of plants, mainly by influencing phytohormone activity and sugar metabolism (Kulma and Szopa 2007). Overexpression of dopamine receptor in potato can increase the levels of soluble sugars, which are the major carbon source that plant hosts deliver to soil microorganisms in a symbiotic relationship (Skirycz et al. 2005). Therefore, we hypothesize that

dopamine may regulate soil microbes by modulating glucose metabolism, thereby alleviating ARD stress in apple.

4.3 Overexpression of MdTYDC mitigated ARD by protecting the photosynthetic system and improving the antioxidant system

MdTYDC transgenic plants had higher shoot and root dry weight than WT. Although some environmental factors restrict plant growth through various physiological processes, photosynthesis is the most severely affected (reflected in changes in biomass production) (Gago et al. 2016). In the present study, ARD caused a rapid decrease in photosynthetic rate, but that decline was significantly slowed in *MdTYDC* transgenic lines in the replant soils. Chlorophyll (Chl) content in plants is an important photosynthetic capacity indicator, a result (and driver) of photosynthesis (Rabinowitch and Govindjee 1965; Field 1991; Field 1995; Rogowski et al. 2019). Chl molecules in the chloroplast thylakoid membrane are the primary harvesters of light energy, and they drive electron transfer reactions in the first stage of photosynthesis. Carotenoids (Car) also play this role through transferred absorbed energy to Chl. The degradation of Chl and Car, and the reduction of their synthesis, leads to abnormal photosynthesis. In the present study, the contents of Chl and Car significantly decreased under replant conditions. All OE lines had higher Chl a and Chl t, and OE-3 and OE-5 lines had higher Chl b and Car than WT. Therefore, *MdTYDC* overexpression mitigated ARD through regulated photosynthesis by inhibiting the degradation of photosynthetic pigment.

Chlorophyll fluorescence is a baseline to examine photosynthetic regulation and plant responses to the environment because of its convenience and sensitivity (Dai et al. 2009). Chlorophyll fluorescence, as a direct proxy for electron transport and photosynthesis, is considered an indicator of environmental stress (Genty et al.1989). In plants, the light energy absorbed by Chl is used to drive electrons in PSII, then by the electron transport chain to PSI to produce ATP and NADPH for the Calvin-Benson cycle (Chou et al. 2020). The maximum photochemical efficiency of PSII (F_V/F_M) as an indicator of photoinhibition and representing the amount of absorbed energy trapped in PSII reaction centers, has been widely studied (Nakamura and Izumi, 2018). In this study, *MdTYDC* transgenic plants showed higher F_V/F_M than WT under replant conditions. The photochemical activity of PSII is reflected by the photochemical quenching coefficient (*qP*), Y (II), and ETR (II); the photochemical activity of PSI is reflected by the Y (I) and ETR (I) (Yi et al. 2018). In this study, Y (I) and ETR (I) was distinctly lower

in WT due to ARD. OE-2 and OE-3 had higher Y (II) compared to WT, under replant conditions. OE-2 and OE-5 had higher Y (I), and all OE lines had higher ETR (I) than WT under replant conditions. Therefore, *MdTYDC* overexpression protected photosynthesis by maintaining the stability of PSI and PSII.

High concentrations of reactive oxygen species (ROS) are toxic to plants and lead to oxidative damage through the oxidation of important components of cells, such as DNA, proteins, and lipids (Demidchik and Vadim 2015; Raja et al. 2017). Previous studies have shown that dopamine-treated plants displayed a lower level of ROS (Gomes et al. 2014). In this study, ARD significantly increased H_2O_2 and O_2^- accumulation in WT plants. However, under replant soil, all OE lines exhibited lower H_2O_2 contents, and OE-2 and OE-5 had lower O_2^- contents than WT plants. Plants possess an antioxidant defense system to restrict ROS levels under stress conditions. In previous studies, continuous cropping decreased activities of SOD, POD, and CAT in *Angelica sinensis* (Oliv.) Diels (Zhang et al. 2010); continuous cropping also significantly decreased SOD activity and increased MDA and the activity of CAT, in *Codonopsis tangshen* (He et al. 2019). Our previous findings also suggested exogenous dopamine leads to lower H_2O_2 contents and higher activities of SOD, POD, CAT, and APX, than non-dopamine plants (Li et al. 2015). In this study, *MdTYDC* overexpression improved activities of SOD, POD, and CAT in the presence of ARD. These components, in turn, detoxify excessive ROS and thus enhance the tolerance of plants to environmental stresses (Choudhury et al. 2017; Yildiztugay et al. 2019). Therefore, *MdTYDC* overexpression detoxified H_2O_2 and O_2^- through improved activities of SOD, POD, and CAT.

Also, the AsA-GSH cycle plays an important role in maintaining the homeostasis of ROS in plants under stress (Rao et al. 1995; Wang et al. 2013). APX, MDHAR, DHAR, and GR are four important enzymes in the AsA-GSH pathway. APX converts H_2O_2 into H_2O and O_2 by catalyzing AsA into DHA, which is then generated to AsA by MDHAR and DHAR in the presence of an electron donor (Li et al. 2018). After DHA regenerates ASA, GSH is oxidized to GSSG, and the GR enzyme can reconvert GSSG to GSH by receiving an electron from NADPH (Roxas et al. 2000; Li et al. 2018). In the present study, pretreatment with dopamine had higher MDHAR and DHAR, and lower DHA and GSSG, compared to the non-dopamine treatment, thus maintaining higher ratios of AsA/DHA and GSH/GSSG under stress (Li et al. 2015). In this study, *MdTYDC* overexpression significantly increased expressions of *MdDHAR1*, *MdcAPX*, *MdcGR*, and *MdMDHAR* under replant conditions. Upregulation of genes of

antioxidant enzymes in the AsA-GSH cycle might be responsible for high rates of AsA/DHA and GSH/GSSG (Cui et al. 2017). These results suggest that the upregulation of these enzymes in the AsA-GSH pathway detoxified H_2O_2 and O_2^- under apple replant conditions.

4.4 Overexpression of MdTYDC mitigated ARD through improved AMF colonization by improving the accumulation of soluble sugars

Under continuous cropping, soil microbial ecology is disrupted, beneficial microorganisms decrease in abundance, and harmful microorganisms accumulate. AMF can stimulate growth, increase disease resistance, and promote community succession and ecosystem stability (Gyuricza et al. 2010; Hu et al. 2017; Cui et al. 2018; Frosi et al. 2018). AMF are the most important beneficial fungi with the largest biomass and the most significant function in the soil and can reduce the pathogenic diseases caused by continuous croppings. Examples of such taxa include *Phytophthora parasitica*, *Rhizoctonia solani*, *Fusarium solani*, and *Ralstonia solanacearum* (Hage-Ahmed et al. 2013; Lewandowski et al. 2013; Vos et al. 2013). Ectomycorrhizal hyphae of AM fungi can help host plants absorb nutrients and water from the soil, drive nutrient cycling, and strengthen the relationship between the soil and host plants (Hodge and Fitter 2010; Laparre et al. 2013). In this study, overexpression of *MdTYDC* improved AM symbiosis in replant soils.

In the symbiotic association between AMF and plant roots, the fungal partner helps plant obtain mineral nutrients, while the host provides carbohydrates for the fungus (Pfeffer et al. 1999; Bucking and Heyser 2001; Bago et al. 2002; Martin et al. 2005; Lanfranco et al. 2016). A previous study found that hexoses had a strong effect on fungal metabolism and development after mycorrhiza formation (Nehls et al. 1998). AM symbiosis promotes sucrose to flow from symbiotic plants to AMF, and then sucrose is decomposed into glucose and fructose before it can be absorbed and utilized by AMF (Blee and Anderson 2002; Heinemeyer et al. 2006; García-Rodríguez et al. 2007). Catecholamines have a positive correlation with soluble sugars and a negative correlation with starch (Swiedrych et al. 2004b). Overexpression of the dopamine synthesis gene in potatoes also increases soluble sugar content (Swiedrych et al. 2004). Increased expression of human dopamine receptors in potatoes results in increased catecholamine content and is accompanied by increases in sucrose, glucose, and fructose (Skirycz et al. 2005b). In the present study, the contents of sucrose and glucose in *MdTYDC* transgenic

lines were higher than WT. Therefore, results suggested that overexpression of *MdTYDC* improved AMF colonization by improving the accumulation of sucrose and glucose.

5. Conclusions

In this study, 100 μ*M* dopamine alleviated ARD stress in apple plants. Exogenous dopamine mitigated ARD through regulated element accumulation and improved soil enzyme activity. In the presence of dopamine, changes in the composition of bacterial and fungal communities demonstrated that various biotic factors are most likely to cause this complicated disease. In replant soils, changes associated with dopamine suggest that dopamine alleviated the effects of ARD and promoted seedling growth. Also, overexpression of *MdTYDC* alleviated ARD stress in apple plants. *MdTYDC* transgenic plants mitigated ARD by inhibiting the degradation of photosynthetic pigment, maintaining the stability of PSI and PSII, and improving the antioxidant system. Moreover, overexpression of *MdTYDC* mitigated ARD through enhanced AMF colonization by improving the accumulation of soluble sugars. These results provide new insights into a method that can be used to protect a valuable fruit crop in replant soils.

Supplementary Data

Supplementary Data for this article are available at Tree Physiology Online.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure 1. Effects of dopamine on N, P, and K concentrations in apple leaves (A, D, and G), stems (B, E, and H), and roots (C, F, and I) when plants were grown in control and replant soil. For each element and tissue type, different letters indicate significant differences at $P < 0.05$ (Tukey's test). CK, plants grown with control soil; DA, dopamine control, control soil supplemented with 100 μ*M* dopamine; ACK, plants grown in replant soil; ADA, a combination of replant soil plus 100 μ*M* dopamine treatment.

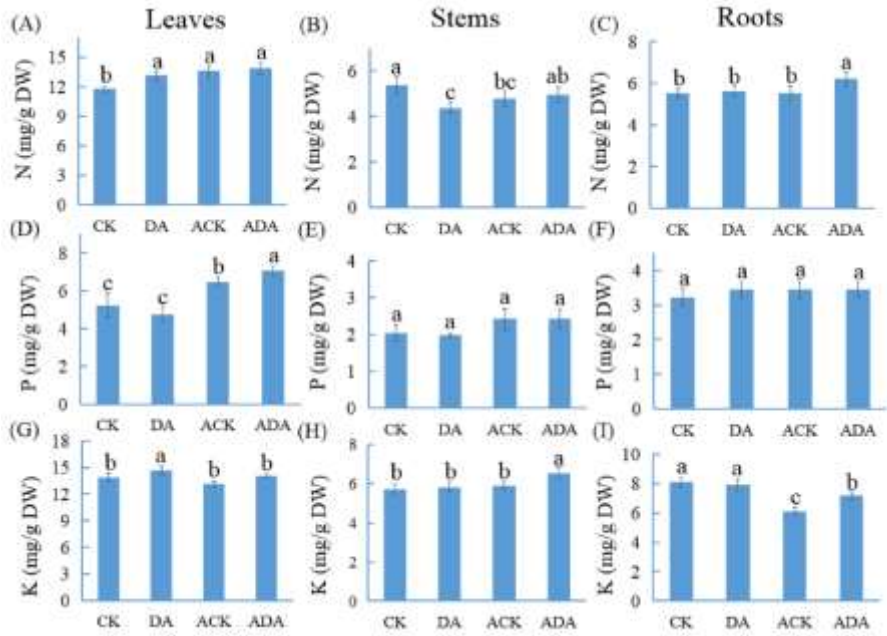

Figure 2. Effects of dopamine on the activity of soil enzymes: invertase (A), urease (B), proteinase (C), and phosphatase (D). Different letters indicate significant differences at $p < 0.05$ (Tukey's test). CK, plants grown with control soil; DA, dopamine control, control soil supplemented with 100 μ*M* dopamine; ACK, plants grown in replant soil; ADA, a combination of replant soil plus 100 μ*M* dopamine.

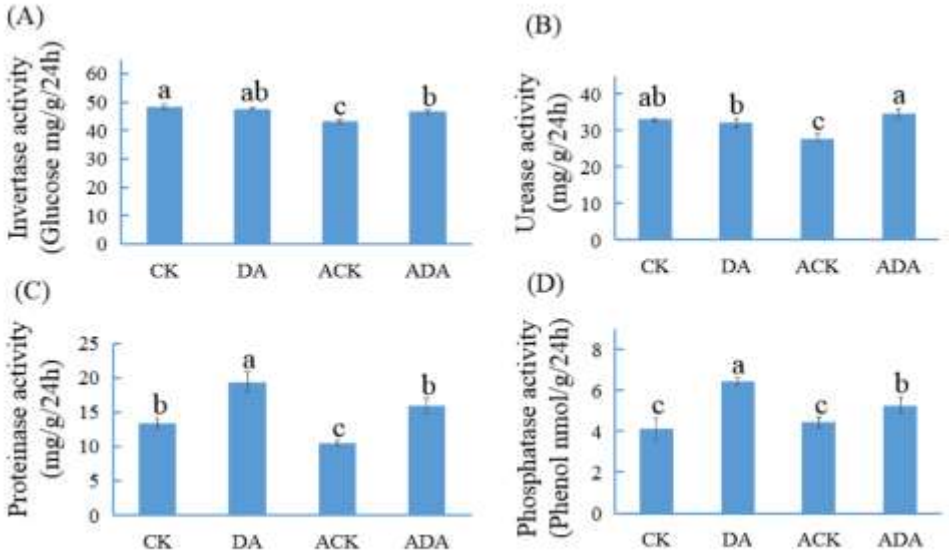

Figure 3. The relative abundance of bacterial communities at phylum (A) and class (B) levels, in the presence or absence of dopamine, based on relative read abundance. Sequences that could not be classified into any known group are labeled as "Others." Each sample is based on the average of three replicates.

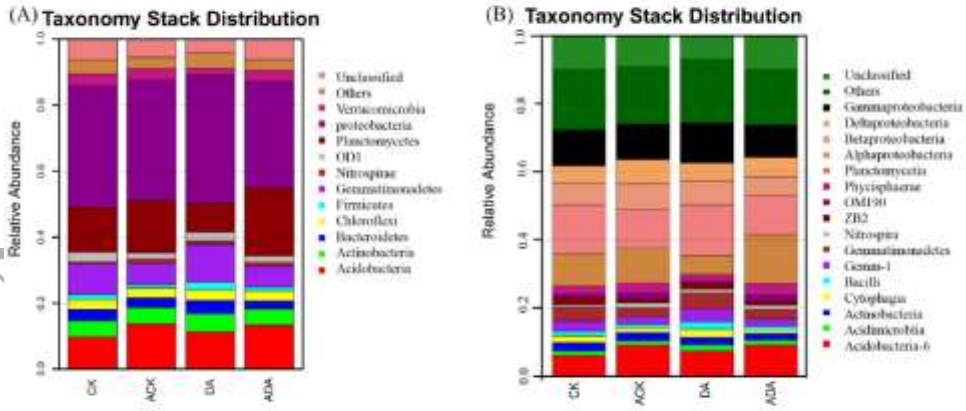

Figure 4. The relative abundance of fungal communities at the genus level, in the presence or absence of dopamine, based on relative read abundance. Each sample is based on the average of three replicates.

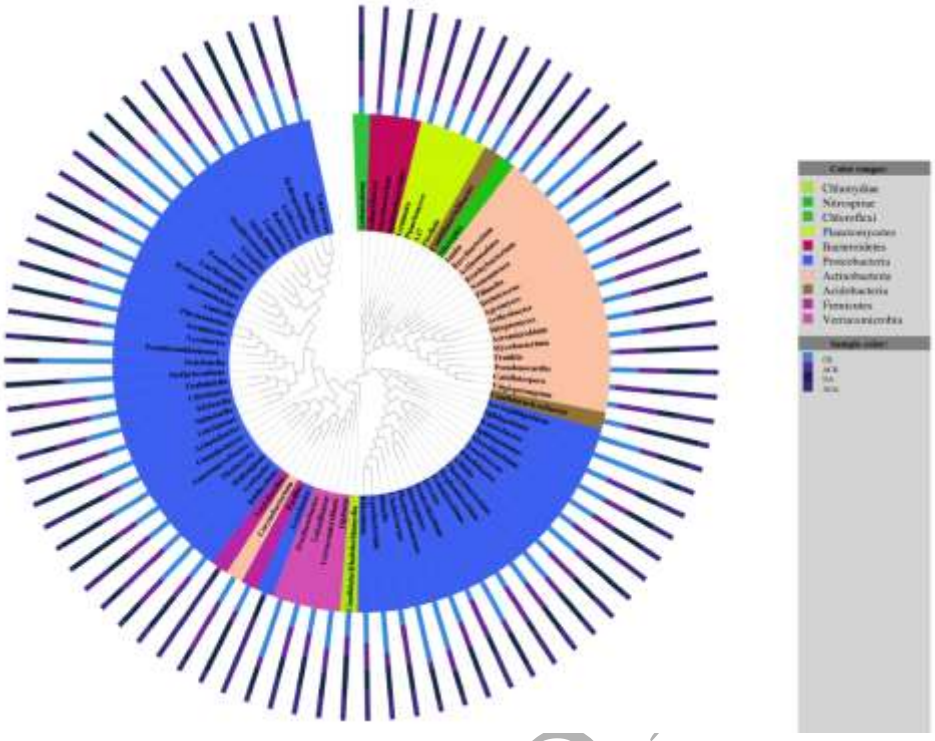

Figure 5. The relative abundance of fungal communities at phylum (A) and class (B) levels, in the presence or absence of dopamine, based on relative read abundance. Sequences that could not be classified into any known group are labeled "Others." Each sample is based on the average of three replicates.

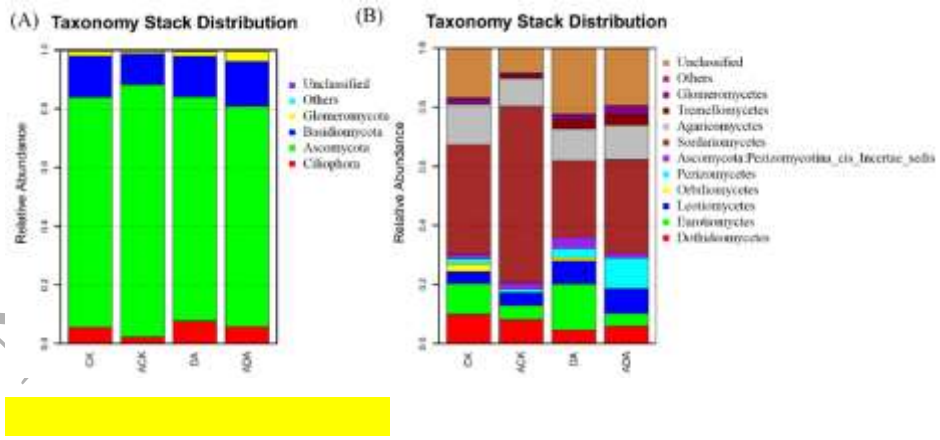

Figure 6. The relative abundance of fungal communities at the genus level, in the presence or absence of dopamine, based on relative read abundance. Each sample is based on the average of three replicates.

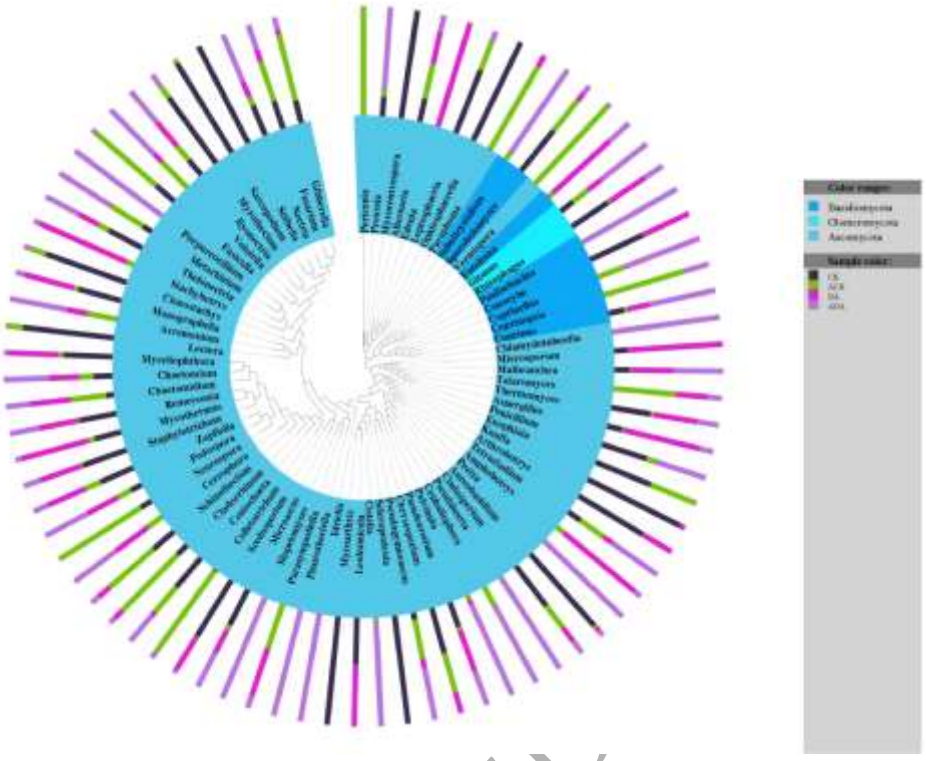

Figure 7. Quantification of dopamine content in transgenic apple leaves (A). The expression of *MdTYDC* in leaves (B) and roots (C). Phenotypes of WT and *MdTYDC* transgenic apple plants under replant conditions (D). WT, wild-type; OE-2, OE-3, and OE-5, *MdTYDC*-transgenic apple lines. Different letters indicate significant differences at $P < 0.05$ (Tukey's test).

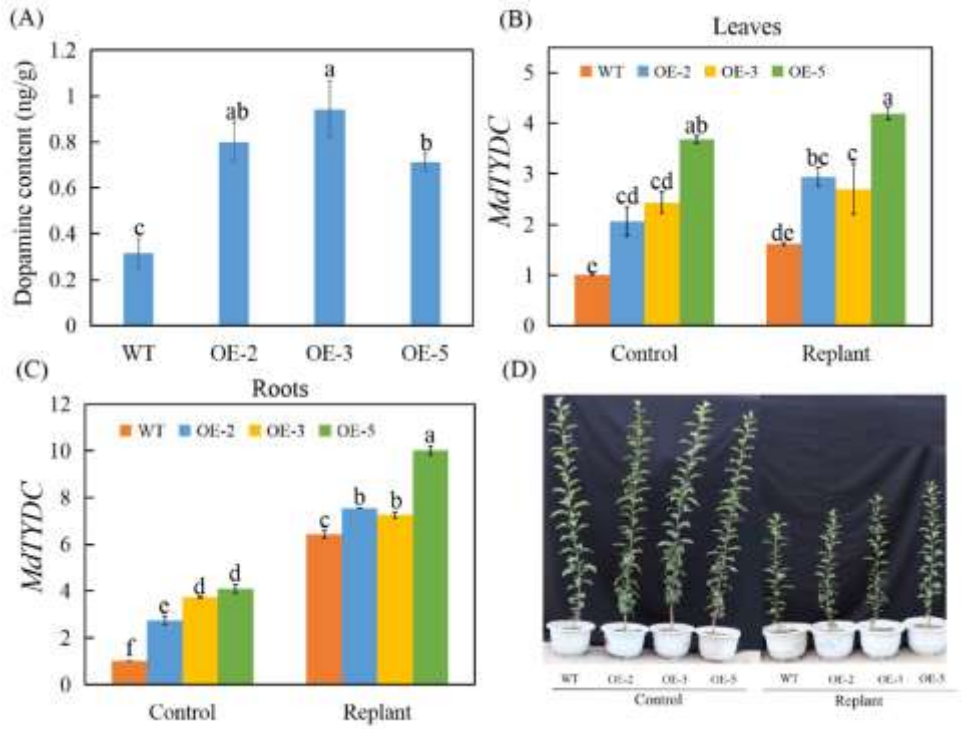

Figure 8. Changes in Chl a (A), Chl b (B), carotenoid (Car; C), total chlorophyll (Chl t; D), P_n (E), g_s (F), and Ci (G) under replant conditions. Different letters indicate significant differences at $P < 0.05$ (Tukey's test).

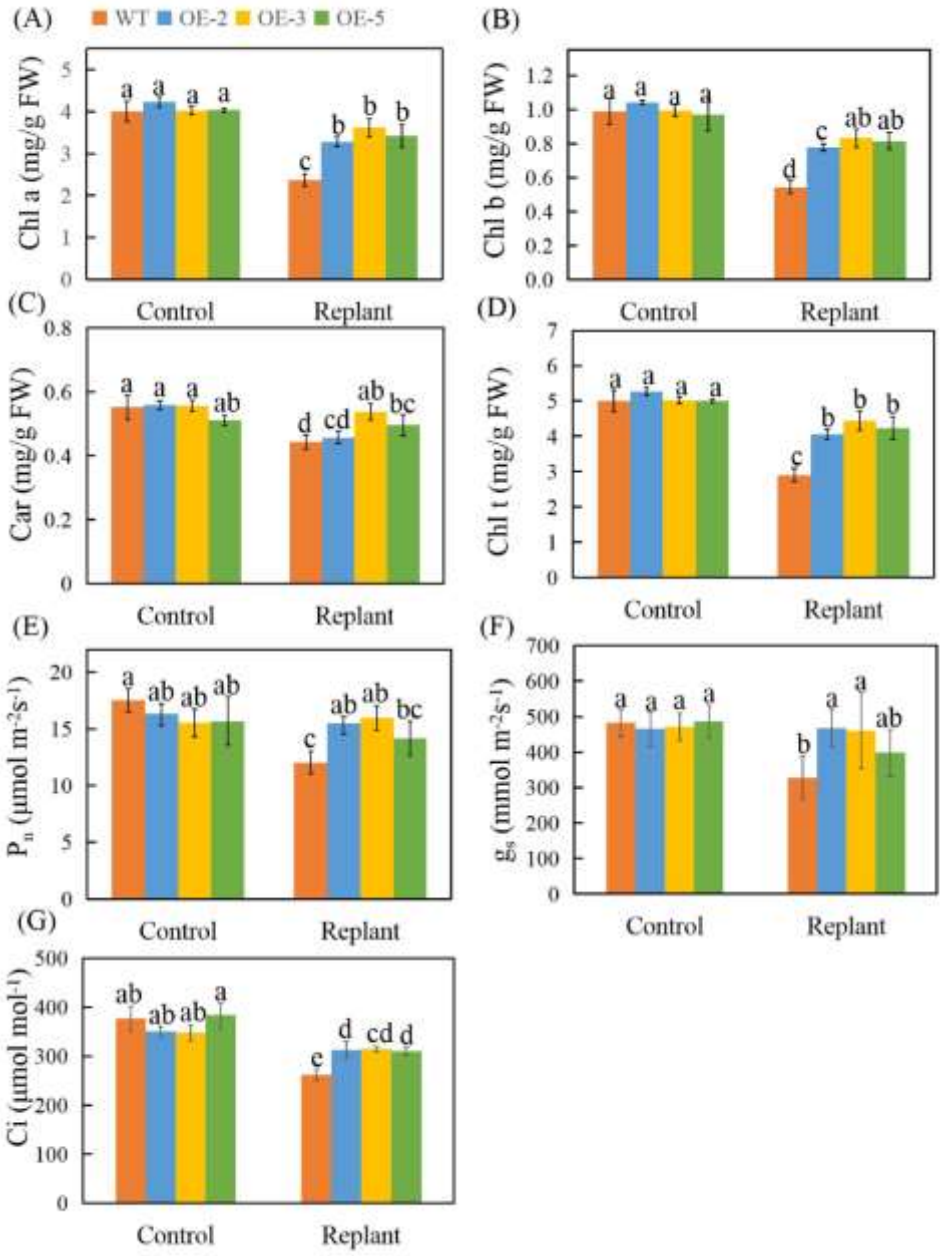

Figure 9. Effects of apple replant disease on the activity of PSI and PSII in apple leaves. (A) Chlorophyll fluorescence images and (B) F_V/F_M ratios of WT and transgenic plants. The color code in the images ranges from 0 (black) to 1.0 (red). Y(I), the effective quantum yield of PSI (C); Y(II), the effective quantum yield of PSII (D); ETR (I), the electron transport rate of PSI (E); ETR (II), the electron transport rate of PSII (F). Different letters indicate significant differences at $P < 0.05$ (Tukey's test).

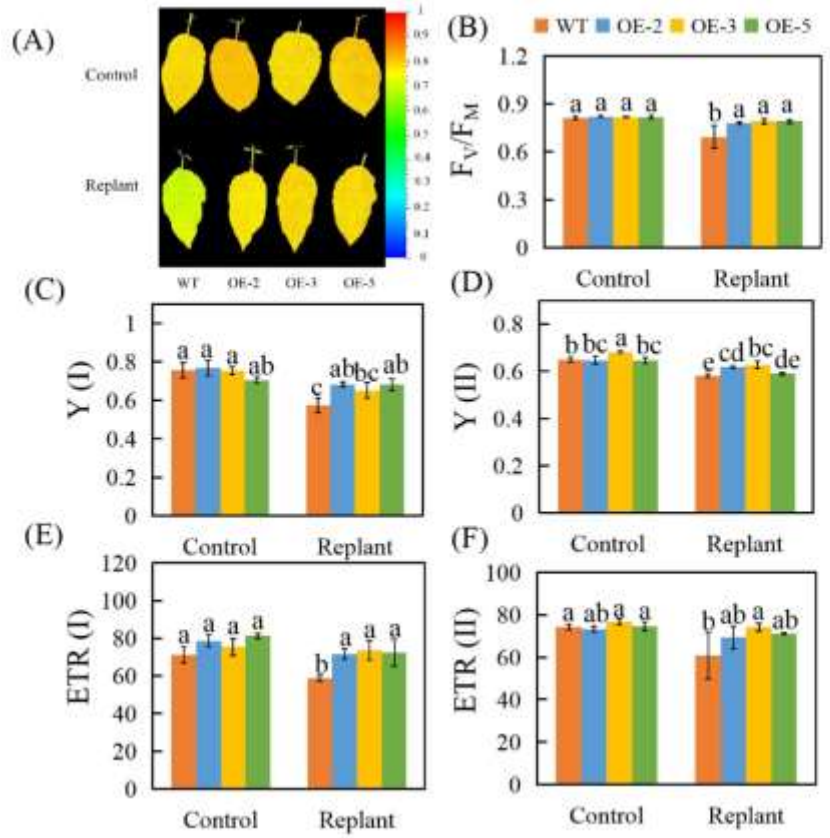

Figure 10. Effects of apple replant disease on the content of H_2O_2 (A) and O_2^- (B), and the activity of SOD (C), POD (D), and CAT (E) in roots of WT and *MdTYDC* transgenic apple plants. Different letters indicate significant differences at $P < 0.05$ (Tukey's test). Heatmap showing expression levels of *MdDHAR1*, *MdcAPX*, *MdcGR*, and *MdMDHAR* in roots (F).

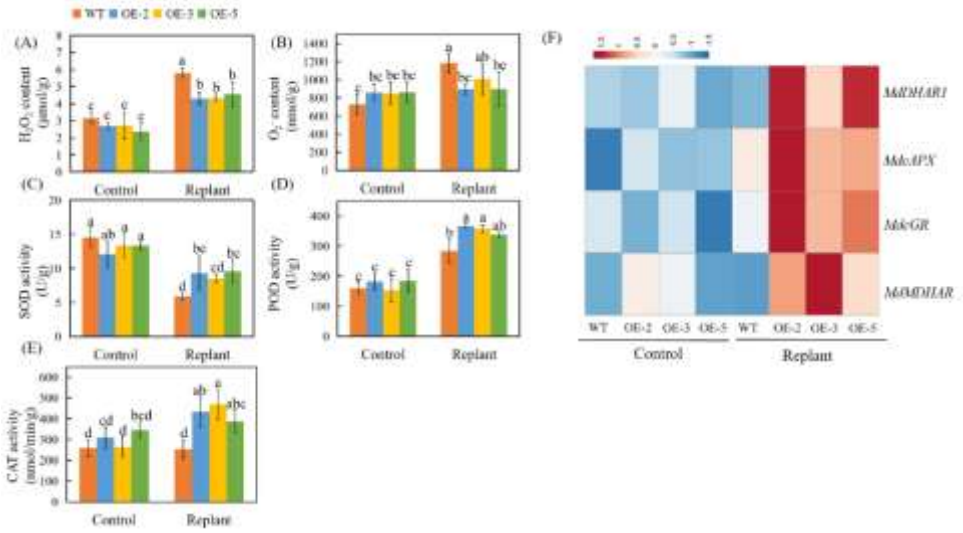

Figure 11. Effects of apple replant disease on AMF colonization (A), sucrose (B), glucose (C), and fructose (D) in roots of WT and *MdTYDC* transgenic apple plants. Different letters indicate significant differences at $P < 0.05$ (Tukey's test).

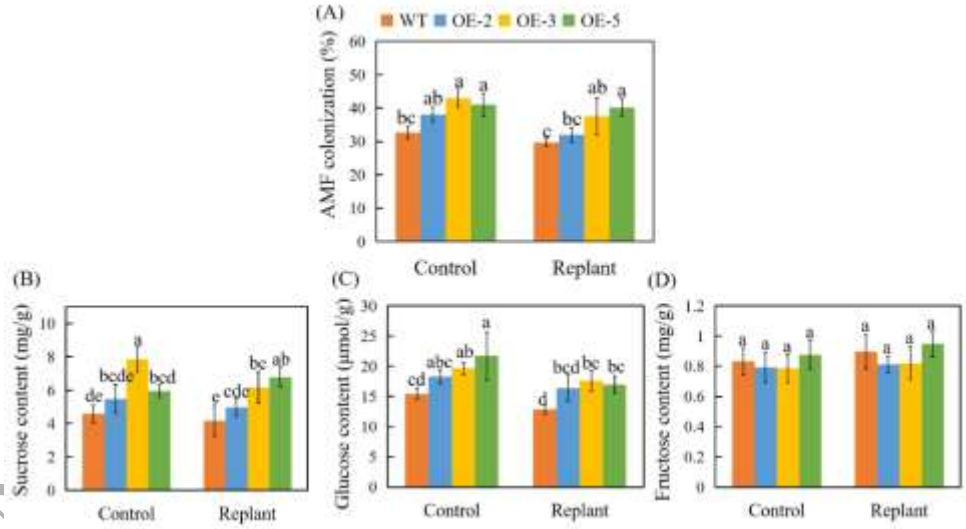

Table 1 Effects of 100 μ*M* dopamine pre-treatment on growth parameters under replant conditions

	Root dry weight (g)	Shoot dry weight (g)	Plant height (cm)	Plant stem diameter (mm)	Leaf area (mm2)
CK	62.56±3.59a	82.73±7.46a	156.45±3.90a	10.73±0.58a	3949.35±246.03a
ACK	32.84±3.51c	33.83±3.07d	121.09±4.29c	7.27±0.31d	2529.05±193.38c
DA	58.50±4.61a	73.55±1.64b	156.80±4.42a	9.44±0.71b	3974.96±245.70a
ADA	44.40±2.52b	51.88±5.84c	141.64±3.31b	8.73±0.48c	3231.39±172.10b

CK, plants grown with control soil; ACK, plants grown in replant soil; DA, dopamine control, control soil supplemented with 100 μ*M* dopamine; ADA, a combination of replant soil plus 100 μ*M* dopamine. All values are given as mean ± SD. Different letters indicate significant differences at $p < 0.05$ (Tukey's test).

Table 2 Values of microbial diversity indices of different groups

Sampling sites	sample	OTUs	Chao	Ace	Shannon	Coverage
Bacterial communities	CK	10699±972a	21529±2112a	26128±2405a	8.05±0.06ab	0.92±0.01a
	DA	9630±1622a	19463±2209a	24349±3270a	7.90±0.19b	0.92±0.01a
	ACK	11038±470a	21564±1540a	26452±2636a	8.19±0.04b	0.91±0.00a
	ADA	10046±737a	19732±1055a	24025±1689a	8.11±0.05ab	0.92±0.00a
Fungal communities	CK	843±75.44a	2330±319a	3975±319a	4.14±0.13b	0.99±0.00a
	DA	889±100.90a	2259±339a	3816±564a	4.06±0.28b	0.99±0.00a
	ACK	1068±235.81a	2455±298a	4323±721a	3.95±0.10b	0.99±0.00a
	ADA	957±60.91a	2183±266a	3357±325a	4.62±0.15a	0.99±0.00a

All values are given as mean ± SD. Different letters indicate significant differences at $p < 0.05$ (Tukey's test)

Table 3 Growth parameters under replant conditions

Strains	Plant height (cm)		Shoot dry weight (g)		Root dry weight (g)	
	Control	Replant	Control	Replant	Control	Replant
WT	132.63 ± 7.09 a	65.88 ± 5.50 b	33.29 ± 7.09 a	6.10 ± 2.27 b	10.1 0± 2.9 a	3.34 ± 1.08 b
OE-2	125.65 ± 5.77 a	66.80 ± 4.97 b	37.41 ± 4.91 a	10.89 ± 2.26 a	13.57 ± 1.98 a	7.42 ± 1.96 a
OE-3	127.44 ± 7.63 a	80.21 ± 4.89 a	32.01 ± 6.77 a	14.58 ± 2.48 a	14.13 ± 1.57 a	6.12 ± 1.37 a
OE-5	124.47 ± 4.56 a	84.42 ± 6.19 a	36.51 ± 2.95 a	13.40 ± 1.73 a	14.14 ± 2.69 a	7.60 ± 0.78 a

WT, wild-type; OE-2, OE-3, and OE-5, *MdTYDC*-transgenic apple lines. All values are given as mean ± SD. Different letters indicate significant differences at $p < 0.05$ (Tukey's test)