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Relationship between foliar endophytes and apple cultivar disease resistance in an organic orchard

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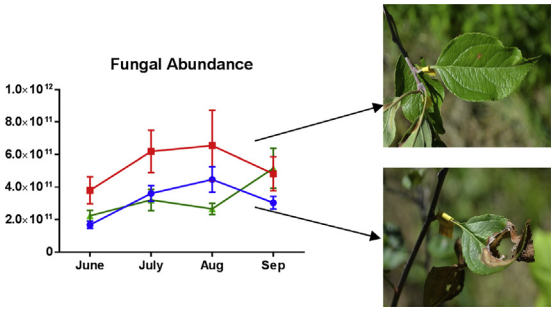
GRAPHICAL ABSTRACT

The cultivar resistant to Alternaria leaf spot had higher fungal abundance than susceptible cultivars when leaves were inoculated with spores (June 25).



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ABSTRACT

Many studies have shown that foliar endophytes enhance host resistance to leaf pathogens. Although plants show large genotype-related intraspecific variation in pathogen resistance, few studies have examined whether disease resistance is also influenced by the endophyte community. In the present study, we tested the hypothesis that apple cultivar differences in leaf pathogen resistance are related to their respective foliar endophyte communities. We inoculated spores of Alternaria leaf spot (*Alternaria mali*) in an organic orchard that had not been exposed to any chemical pesticide for over 30 years, and evaluated leaf damage in 18 apple trees belonging to three cultivars with differing levels of resistance. The richness, composition, and abundance of the foliar endophyte communities in the same 18 trees were also examined using terminal restriction fragment length polymorphism. We found that cultivar resistance to lower leaf damage correlated significantly with the abundance of endophytic fungal species. These results suggest that endophytic fungi can promote leaf spot resistance in apple cultivars.

1. Introduction

The application of fungicides is currently the primary method used to control plant pathogens. However, many publications have discussed the emergence of pathogens with high levels of fungicide resistance (Sanssené et al., 2011; Selim et al., 2011). Therefore, the development of alternative environment-friendly control methods is required.

The leaves of higher plants harbor endophytic microbes (Peñuelas and Terradas, 2014; Vandenkoornhuyse et al., 2015). Several studies have shown that these foliar endophytes can enhance host pathogen resistance (Arnold et al., 2003; Porras-Alfaro and Bayman, 2011; Mejia et al., 2014; Ritpitakphong et al., 2016). The effects of endophyte

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infection on host resistance are strongly influenced by the inoculation method and the experimental conditions. Laboratory-based *in vitro* assays do not adequately model the pathogen resistance of the whole plant because they do not involve the host plant immune response (Mejia et al., 2014; Pieterse et al., 2014; Raghavendra and Newcombe, 2013). However, inoculation studies of endophytes and pathogens *in planta* can produce inconsistent results that depend on the endophyte species used and the order of inoculation employed (Adame-Álvarez et al., 2014). The actual effects of foliar endophytes on host plant disease resistance are more likely to be identified by natural field testing, where diverse microbial species—including pathogens—establish communities within leaves. Organic farms that have not been exposed to chemical pesticides provide an environment that allows the development of natural endophyte communities within leaves; this provides a good opportunity to test the effects of foliar endophytes on the disease resistance of host plants.

The diversity and taxonomic composition of foliar endophyte communities vary greatly with plant age, host species identity, and location (Arnold and Herre, 2003; Coleman-Derr et al., 2016; Hoffman and Arnold, 2008). In addition, genotypic differences between the host plants have significant effects on foliar endophyte communities (Bulgarelli et al., 2012; Edwards et al., 2015; Lundberg et al., 2012; Sapkota et al., 2015; Wagner et al., 2016). However, the influence of foliar endophytes on the disease resistance of cultivars of a given species has not been examined previously. Apple cultivars (Malus pumila *var. domestica*) show large differences in resistance to Alternaria leaf spot, which is caused by *Alternaria mali* (Kohmoto et al., 1977). The current study examined the relationships between foliar endophyte communities and leaf pathogen damage in three apple cultivars with different levels of resistance to Alternaria leaf spot in an organic orchard. We quantitatively evaluated the extent of leaf damage in the three cultivars following inoculation with *A. mali* spores. Simultaneously, we examined the richness, composition, and abundance of the bacterial and fungal endophyte communities in the leaves using the molecular fingerprinting method, terminal restriction fragment (T-RF) length polymorphism (T-RFLP). To investigate the relationship between the foliar endophyte community and host disease resistance, the following specific questions were addressed: Does the bacterial and fungal composition of the foliar endophyte communities differ in the three cultivars included in this study? Is there any relationship between foliar endophyte community and resistance to Alternaria leaf spot in these three cultivars?

2. Material and methods

2.1. Study site

This study was conducted in an organic 0.8-ha apple orchard in Hirosaki, northern Japan (40.64 N, 140.36 E). The apples in this orchard had been cultivated without any fertilizer or agrochemicals of any kind for > 30 years. We studied three apple cultivars: Kogyoku (K), Fuji (F), and Orin (O). Cultivar K is more resistant to Alternaria leaf spot than the F and O cultivars (Kohmoto et al., 1977). The experimental units included six trees from each of these three cultivars, selected from different places within the orchard.

2.2. Inoculation study

A. mali was cultured on petri dishes containing 30% potate sugar agar (PSA) medium in a controlled environment chamber at 25 °C for 90 days; spore formation was then induced by exposure of the cultures to ultraviolet light for 30 days. The spores were collected and suspended in sterilized water to achieve a final concentration of 2×10^5 ml^{-1}. This solution was applied to three young and fully expanded leaves on each tree on June 25, 2013, using medical adhesive plasters; these were 22 mm in diameter, with unwoven 10-mm cloth squares in the center (Careleaves CL16C, NICHIBAN, Tokyo, Japan). The spore solution (100 µl; 2×10^4 conidia) was applied to the cloth of the adhesive plaster, and the plaster was then attached to the abaxial surface of the leaf. Each inoculated leaf was covered with a plastic bag (60 mm × 85 mm), which contained a piece of water-saturated filter paper to maintain an appropriate level of humidity. The plasters were removed from the leaves 2 days after inoculation. In September, the disease damage was measured using digital images of each leaf, captured with a scanner. The Alternaria leaf spot lesion areas were measured using Image J software (National Institutes of Health, Bethesda, Maryland, USA). The area of the lesion was expressed as a proportion of the whole leaf area; this represented the disease damage index.

2.3. Leaves' microbial community analysis

2.3.1. DNA extraction

Three leaves were sampled from each of the 18 experimental apple trees on each of the four harvest dates (June 27th, July 26th, August 26th, and September 30th) for the analysis of foliar endophyte communities. The leaves were washed in sterilized water in a sonication bath for 2 min to remove microbes from the leaf surface. Four 2-cm^2 leaf disks were cut out from each single leaf for DNA extraction using the method described by Varadarajan and Prakash (1991); the DNA was then kept at -20 °C until further analysis.

2.3.2. Real-time PCR

The DNA extracted from the leaf samples was analyzed by real-time PCR using a Chromo 4 DNA Engine (Bio-Rad, Forster City, CA, USA). The primers used were FR1 (AICCATTCAATCGGTAIT) and FF390 (CGATAACGAACGAGACCT) for fungal DNA, and 799f (AACMGGATTAGATACCCKG) and pHr (AAGGAGGTGATCCAGCCGCA) for bacterial DNA. These bacterial primers were reported to avoid the amplification of plastid DNA (Ferrando et al., 2012). Sequence analysis of 40 clones of amplified DNA revealed the absence of any plastid DNA derived from mitochondria or chloroplasts. Quantitative real-time PCR was performed in a total volume of 10 µl, containing 5 µl of iQ SYBR Green Supermix (Bio-RAD), 0.5 mM primers (forward and reverse), and 1 µl of template DNA. As a standard curve constructed using DNA from a single species causes a biased estimation of copy number in environmental samples composed of diverse species (Towe et al., 2010), the calibration curve employed in the present study was prepared using the DNA of a whole community, rather than the DNA of a single endophytic species. The community DNA was amplified by PCR and then purified using PCR quick-spin™ (iNtRON Biotechnology, Seongnam, Korea) prior to determining its concentration using NanoDrop 2000 (ThermoFisher Scientific, Wilmington, DE, USA). The standard curve was created using a dilution series of this target DNA (10^3 copies µl^{-1} to 10^8 copies µl^{-1}); the copy number was calculated using the molecular weight of the amplicon. The copy numbers present in each sample were then calculated using the threshold cycle (C_T) value, and expressed per leaf area (m^2). To avoid any systemic instrument error, the leaf samples were analyzed in a single 98-well plate assay, which included the calibration standards.

2.3.3. T-RFLP analysis

The DNA extracted from the leaf samples was analyzed by PCR using primers for ribosomal RNA (rRNA) genes. For fungi, ITS1F (CTTGGTCATTTAGAGGAAGTAA) labeled with 6-FAM™ and ITS4 (TCCTCCGCTTATTGATATGC) were employed, and for bacteria, 799F (AACMGGATTAGATACCCKG) labeled with 6-FAM™ and 1492R (GGYTACCTTGTTACGACTT) were employed. The 25-µl reaction mixture contained 1 µl of template DNA (a 1:10 dilution of the original extract), 2.5 µl of 10 × buffer, 3 µl of 25-mM $MgCl_2$, 2 µl of a 2.5 mM concentration of the deoxynucleoside triphosphates, 1 µl of each primer (12.5 µM), and 2.5 U of TEMPase Hot Start DNA polymerase (Amplicon, Skovlunde, Denmark). The DNA was denatured by incubation at 95 °C