ENDOPHYTE-MEDIATED CONTROL OF FUNGAL PATHOGENESIS IN *ARABIDOPSIS THALIANA*

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Graduate School of Biological Sciences　Doctoral Thesis Abstract

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ABSTRACT

Plants constantly associate with a vast diversity of microbes that range from harmful pathogens to beneficial endophytes. Pathogen infection can cause discernible disease symptoms. In contrast, some of the endophytes provide host plants with fitness benefits such as growth promotion, abiotic stress adaptation, and protection from pathogen infection. Plants in nature are expected to be constantly associated with both beneficial and potentially harmful microorganisms, without discernible disease. However, it is not currently clear how this seemingly peace status is maintained in the presence of potential pathogens. This study investigates the impact of a newly isolated endophytic fungus as a host protective agent against root fungal pathogens.

Colletotrichum, the large ascomycete genus, is one of the most economically important groups of plant pathogens. *Colletotrichum* fungi cause anthracnose disease in a wide range of economically important crops. It has been also recently reported that some of *Colletotrichum* species promote plant growth in nutrient-limiting conditions. Therefore, the *Colletotrichum* genus provides key fungal resources for studies on diverse infection strategies ranging from parasitic to potentially mutualistic strategies.

I put an initial focus on two closely related fungal isolates of *Colletotrichum* from healthy radish (*Raphanus sativus*) roots in an open field in NAIST. I found that one acts as a pathogen (CgP) in the reference plant *A. thaliana*, while the other acts as an endophyte (CgE) that colonizes plants without causing disease symptoms. Remarkably, co-infection of CgP and CgE on *A. thaliana* roots has revealed that CgE restricts CgP growth in roots and protects the host from damage caused by CgP, importantly without causing discernible penalty on plant growth. Moreover, the CgE-mediated host protection is also effective against another distantly related *Colletotrichum* pathogen, *C. incanum*. Therefore, these results indicate that CgE identifies a novel fungal endophytic isolate of *Colletotrichum* with host protection function against different root fungal pathogens.

To investigate the mechanisms underlying the endophytic lifestyle and host protection ability of CgE in Arabidopsis roots, I conducted transcriptome analysis on roots colonized with CgE in the presence and absence of CgP. The overall profile of plant transcriptome following co-inoculation is highly similar to that following single CgE inoculation, but is far diverged from that following single CgP inoculation. Together with CgE-mediated disease protection stated above, this suggests that CgE effectively suppresses CgP infection and/or its caused host responses.

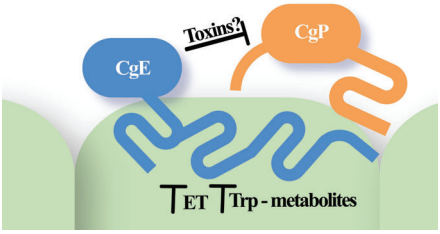
It should be also noted that the overall transcriptome in CgE-inoculated plants is largely indistinguishable with or without CgP co-inoculation, implying that massive activation of defense-related genes is not associated with CgE-mediated CgP suppression.

Notably, CgE expresses pathogenesis in *cyp79B2 cyp79B3* plants, which are defective in the cytochrome P450s that mediate an initial and essential step in the production of tryptophan-derived antifungal metabolites, such as glucosinolates. CgE-mediated plant protection was not either observed in *ein2 sid2 dde2 pad4* quadruple mutants, which simultaneously lack key components of ethylene (ET) signaling (EIN2), salicylic acid biosynthesis and signaling (SID2 and PAD4, respectively), and jasmonic acid biosynthesis (DDE2). Analyses on triple and single *A. thaliana* mutants for these defense components have revealed that the ET pathway is required for the establishment of the beneficial interaction with CgE. In the absence of ET signaling, CgE massively infects the roots and eventually kills the host plant, suggesting that the proper control of fungal colonization mode by the host ET pathway is critical for the beneficial function.

To further investigate into fungal responses during competition between CgE and CgP, I have determined the whole genome sequences of both fungi and have analyzed the transcriptome data from the fungal side. Comparative genomic analysis between CgP and CgE reveals that two fungi belong to the same *gloeosporioides* clade but are different species. Owing to the differences between the two species, I successfully discriminated the majority of CgE-derived RNA-sequencing reads from CgP-derived reads in their co-inoculated root samples. Comparative transcriptome analysis of the co-inoculated samples and the corresponding individually-inoculated samples resulted in the identification of more than 500 CgE genes that are specifically up-regulated in co-inoculation with CgP, indicating that large-scale transcriptome reprograming occurs during fungus–fungus competition in the roots. This is in sharp contrast with the less distinguishable transcriptome profiles of the host roots between CgE single inoculation and its co-inoculation with CgP. Interestingly, CgE genes for the biosynthesis of several fungal toxins (e.g., botrydial) are strongly up-regulated specifically in the co-inoculated samples, raising the possibility that these CgE fungal toxins contribute to CgP growth restriction in the roots.

Plant-microbe interactions are substantially affected by nutrient conditions. Interestingly, CgE does not display the aforementioned endophytic interaction mode with the host under inorganic phosphate (Pi) limiting conditions, but instead displays a pathogenic interaction mode to kill the host.

In summary, my study has developed a new model to learn how fungal endophytes mediate plant protection from pathogens and gained initial insight into this important question. This model is expected to deepen our understanding of the molecular basis and evolutionary aspects of the differentiation between endophytes and pathogens.



Graphical Abstract: A working model for control of endophytic CgE lifestyle and its host protection

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ABBREVIATIONS

4MI3G	4-methoxy indolyl-3-methyl glucosinolates
4OHICN	4-hydroxyindole-3-carbonitrile
BAM	Binary Alignment Map
Cam	Camalexin
CERK	*CHITIN ELICITOR RECEPTOR KINASE*
CgE	Endophytic *Colletotrichum gloeosporioides*
CgP	Pathogenic *Colletotrichum gloeosporioides*
Col-0	Columbia-0
CYP79B2	*CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2*
CYP79B3	*CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 3*
DDE2	*DELAYED DEHISCENCE 2*
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
EIN2	*ETHYLENE INSENSITIVE 2*
ET	Ethylene
FLS2	*FLAGELLIN-SENSITIVE 2*
GFP	Green Fluorescent Protein
GM	Genetically Modified
GO	Gene Ontology
hpi	Hours post inoculation
I3A	Indole-3-ylmethylamine
I3C	Indole-3-carbinol
IAOx	Indole-3-acetaldoxime
IGs	Indole-glucosinolate derivatives
ISR	Induced Systemic Resistance
ITS	Internal Transcribed Spacers

JA	Jasmonic Acid
LRR	Leucine-Rich Repeat
MAMPs	Microbe-Associated Molecular Patterns
MAPK	Mitogen-Activated Protein Kinases
MS	Murashige and Skoog medium
PAD3	*PHYTOALEXIN DEFICIENT 3*
PAD4	*PHYTOALEXIN DEFICIENT 4*
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PEN2	PENETRATION2
PI	Propidium Iodide
PR	Pathogenesis-Related
PRRs	Pattern Recognition Receptors
PSR	Phosphate Starvation Response
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain reaction
RLKs	Receptor Like Kinases
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SID2	*SALICYLIC ACID INDUCTION DEFICIENT 2*
Suc	Sucrose
Trp	Tryptophan

INTRODUCTION

Plants live in nature surrounded by rich and diverse microbial communities. Plants have developed a complex defense system against pathogenic microorganisms. At the same time, plants have accepted beneficial microorganisms and let them live inside as endophytes probably for mutual benefits. Plants provide photosynthetically fixed carbon (e.g. root exudates, litter) to microbes. In addition to its direct transfer to microbes within the host, it has been also assumed that up to 20-50% of photosynthetic carbon is secreted from roots to the rhizosphere, of which the majority is utilized by microbes (Jones et al., 2009; Jacoby et al., 2017; Baettie 2018). In exchange, endophytes contribute to host nutrient uptake, and also to host protection from pathogens (Varma et al., 2012; Hiruma et al., 2018). So far, most of our knowledge for beneficial microbial functions is derived from studies on bacterial endophytes except a few beneficial fungi, such as arbuscular mycorrhizal fungi. In contrast, relatively little is known about beneficial functions of fungal endophytes, despite their richness and diversity in the root ecosystems. In this thesis, I study beneficial features of a fungal endophyte that is newly isolated from healthy Brassicaceae plants grown in the field.

Limitation of using chemical pesticides and genetically modified plants for plant protection

Plant diseases exhibit severe problems on agricultural production worldwide. Many diseases result in damage in the appearance of the products and the loss of the yield. If plants in the field are infected by a pathogen, it is very important to get rid of pathogens from infected plants to protect still uninfected plants. To restrict the emergence of pathogens, there are several prevailing methods for these days. Chemical approach is very effective against a wide range of fungal pathogens (Everett et al., 1996). However, it can cause environmental pollutions and is harmful to the human health.

Another way to protect plants is to activate plant immune responses. In the absence of adaptive immunity, plants have evolved an elaborate immune system based on innate immune receptors (Jones & Dangle, 2006). In plants, recognition of non-self or altered-self by pattern recognition receptors (PRRs) leads to a first line of defense responses that restrict pathogen infection (Boller & Felix, 2009; Macho & Zipfel, 2014). PRR ligands include microbial signatures typically conserved within microbes, termed microbe-associated molecular patterns (MAMPs). For example, in Arabidopsis, the Leu-rich repeat (LRR)-receptor like kinases (RLKs) FLS2 recognizes the bacterial flagellin (epitope flg22), and the lysin motif (LysM)-RLK CERK1 underlie recognition of a fungal cell wall component (chitin) (Gómez - Gómez & Boller, 2000; Zipfel et al., 2006; Kaku et al., 2006). Following ligand perception, PRRs form signaling complexes to trigger a set of defense-related outputs. Changes of ion fluxes across the plasma membranes, reactive oxygen species (ROS) bursts, and MAPK activation are typically detectable within a few minutes. They are followed, within several hours to days, by ethylene (ET), and salicylate (SA) production, extensive transcriptional reprogramming, cell wall remodeling, and metabolic changes including biosynthesis of anti-microbial compounds (Boller & Felix, 2009; Segonzac & Zipfel, 2011). The activation of these defense outputs collectively leads to effective immunity against pathogens. Despite the effectiveness, activation of plant immune responses is often associated with plant growth retardation, resulting in the fitness disadvantage in the absence of pathogens (Gurr et al., 2005; Hout et al., 2014). However, although knowledge of plant immune system is rapidly expanding, it remains poorly understood how plants manage this defense-growth tradeoff.

In this respect, recent studies have hinted at new strategies to generate disease resistance in plants without causing much growth penalty. For example, transgenic *Nicotiana*

benthamiana (Tobacco) and *Solanum lycopersicum* (Tomato) plants expressing Arabidopsis EFR that perceive bacterial MAMP EF-Tu has an increased degree of resistance to several bacterial pathogens, although its effects on beneficial endophytes remain to be tested (Lacombe et al., 2010). In addition, Xu et al., 2017 successfully engineered board-spectrum disease resistance in *Arabidopsis thaliana* and rice without discernible trade-off with plant biomass. However, due to the genetically modified (GM) plant issues, these genetic engineering approaches cannot be directly applied to agriculture in most of the countries. To overcome this issue, alternative method(s) not depending on GM approaches will need to be developed for sustainable agriculture.

Some endophytic fungi provide host protection

Endophytic fungi are the fungi that associate with and live within the host plants without harm or causing diseases to the host, at least in a period of the host plant life-cycles (Rodriguez, 2009). Several fungi are reported to show beneficial traits, such as plant growth promotion. For example, the endophyte *Colletotrichum tofieldiae* colonizes roots of *Arabidopsis thaliana* without causing disease symptoms and even promotes plant growth under low-phosphate conditions via transferring phosphorus to the hosts (Hiruma et al., 2016). In addition, *Fusarium oxysporum* has been reported to enhance plant shoot growth via production of volatile compound(s) (Bitas et al., 2015). Some of the fungi are reported to produce special metabolites or chemical molecules such as antimicrobial agents and/or plant growth factors in their hosts (Rodriguez et al., 2008). For example, antifungal compounds, such as several indole derivatives, sesquiterpene, and diacetamide, are produced by *Epichloë festucae* (Yue et al., 2000). Antifungal compounds are presumed to be used during microbe-microbe competition for niches and therefore can be used as biocontrol agents against plant-infecting pathogens (Punja et al., 2003). In addition, endophytic fungi are also reported to protect plants from abiotic stresses such as salt and heat during symbiosis via unknown mechanisms (Rodriguez et al., 2008).

Host protection by endophytes can be largely divided into two ways: direct and indirect effects. Direct effects rely on direct competition between endophytes and pathogens, including mycoparasitism (Brimner et al., 2003) and production of antifungal compounds (Živković et al., 2010; Verma et al., 2007). For example, fungi in the genus *Trichoderma* are widely known to inhibit other fungi through the parasitic infection and secretion of many anti-fungal secondary metabolites (Schuster & Schmoll, 2010; Gupta et al., 2014). Like plants, the primary defense system of fungi also includes resistance against toxic compounds such as secondary metabolites, peptides and proteins which are specifically targeted on the antagonists (Künzler, 2018). These mechanisms are believed to facilitate fungal survival in nature when they compete with others for the shared niches and limited resources.

A competition between two or more fungi could occur in plant hosts. For instance, three *Pisolithus spp*. which associate with Eucalyptus plants can directly compete with each other for limited space in plants. On the other hand, host Eucalyptus plants limit root tip colonization of unwanted or even favored fungal partners, leading to impacting the degree of fungus-fungus competition in plants (Hortal et al., 2017). It has been reported that suppression of disease symptoms by beneficial endophytes such as *Trichodema harzianum* and *Serendipita vermifera* is observed without detectable host transcriptome changes (Morán-Diez et al., 2012; Sarkar et al., 2019). The authors have proposed that plant protection mediated by the endophytes could rely on the ability to infect pathogens as parasites, pointing to a role for microbe-microbe competition in host protection.

As for indirect effects, some fungi activate host plant defense responses and then promote a host ability to prevent infection of plant pathogens. For example, Dupont et al (2015) reported that endophytic *Epichloe festicae* altered host transcriptome during plant-endophytic

association. Most of differentially regulated genes include gene ontology (GO) categories related to host metabolism, responses to stresses and hormonal responses. Furthermore, Su et al., 2013 showed how the endophytic *Harpophora oryzae* provided rice resistance against rice blast fungi (*Magnaporthe oryzae*). In addition, not only fungi themselves, but their metabolites can also induce plant defense responses (Chalfoun et al., 2011). For example, Shen et al., 2018 showed that treatment of a volatile compound, namely indole, induced plant defense responses against necrotrophic pathogens *Fusarium graminearum* and *Fusarium moniliforme* in maize.

However, the molecular basis for fungal protection (competition) strategies or how these strategies are executed and shaped in the host plants remains largely unknown.

Colletotrichum fungal genus is diverged in infection strategies ranging from beneficial to pathogenic.

The large ascomycete genus *Colletotrichum* is one of the most economically important groups of plant pathogens, causing anthracnose disease on a wide range of economically important crops (Agrios, 1988). In 2012, *Colletotrichum* was also nominated as one of the ten most important fungi based on the scientific and economic importance (Dean et al., 2012). In contrast to true obligate biotrophs such as powdery mildews, *Colletotrichum* species can be cultured axenically and can be genetically modified by stable transformation including gene knockout via homologous recombination. This greatly facilitates mutational analysis and critical assessment of gene functions by targeted gene disruption. In addition to this merit, more than ten high quality genome sequences of *Colletotrichum* species are currently available for detailed comparative genomic analysis (O'Connell et al., 2012; Gan et al., 2013 and 2016; Hacquard et al., 2016).

Many *Colletotrichum* species, like *Magnaporthe oryzae*, are hemibiotrophic pathogens, which initially grow biotrophically in living host cells before switching to a destructive necrotrophic phase of infection (Perfect et al., 1999; Koga et al., 2004).

Although most of them are recognized as hemi-biotrophic plant pathogens, recently some of them have been reported as plant endophytes. For example, *Colletotrichum tofieldiae* colonizes the roots of *Arabidopsis thaliana* without causing diseases to promote plant growth under low-phosphate conditions, in part via transferring inorganic phosphate to the host (Hiruma et al., 2016). Isolation of putative endophytic fungi closely related to pathogens, such as *C. tofieldiae*, has been repeatedly reported from different plant species for the last decades (Rodriguez & Redman, 2008), suggesting that potential pathogenesis of these fungi is restricted in nature via unknown mechanisms (Hiruma et al., 2018). Potential benefits of these fungi to plant growth and health remain to be tested.

Phytohormones as the critical regulators of plant defense responses

Phytohormones are main regulators of plant growth and response to environments, including responses to microorganisms. The plant hormones salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) play a central role in the regulation of plant immune responses (Robert-Seilaniantz et al., 2011). SA signaling positively regulates plant defense responses against biotrophic pathogens that feed on living host tissues, while ET/JA generally regulate defense responses against necrotrophic pathogens that kill the host cells and feed on dead tissues (Glazebrook, 2005; Bari & Jones, 2009). All the phytohormone pathways are connected to each other in a complex network to coordinate defense responses (Broekgaarden et al., 2015). Generally, SA- and ET/JA-dependent defenses are typically antagonistic to each other in most cases. However, these defense sectors can compensate each other when they are simultaneously disrupted with mutations in genes encoding isochorismate synthase1 required for SA biosynthesis, called SID2, the SA-related defense regulator PAD4, the allene oxide synthase required for JA biosynthesis, called DDE2 and a master regulator of ET signaling, EIN2.

Therefore, simultaneous disruptions of these defense components in *Arabidopsis thaliana* are required to largely abolish defense responses against various types of pathogens (Tsuda et al., 2009; Cui et al., 2017)

ET controls several processes such as plant growth, flowering, root formation, fruit ripening, seed germination, in addition to plant adaptation to biotic/abiotic stresses (Abeles et al., 2012). ET is not only required for systemic acquired resistance (SAR) (Fu & Dong, 2013), but also for induced systemic resistance (ISR) (Pieterse et al., 2014). Consistent with this, ET has been reported to modulate beneficial associations with microbes (Zamioudis and Pieterse, 2012). For example, *Arabidopsis thaliana* and barley activate ET signaling for the proper colonization of the mutualistic fungus *Piriformospora indica* (Khatabi et al., 2012). On the other hand, ET also works together with JA to restrict growth of beneficial ectomycorrhizal fungi, possibly to optimize the host benefits obtained from this mutualistic plant-fungus interaction (Plett et al., 2014). Arabidopsis mutants defective in ET responses, such as *ein2* and *ein3*/*eil*, fail to establish beneficial interactions with the root endophyte *P. indica* (Camehl et al., 2010). Thus, ET represents a key factor utilized by the plant and/or its partners to build the beneficial interactions.

Control of fungal behaviors by tryptophan-derived secondary metabolites

Plants synthesize a wide array of secondary metabolites with diverse roles, including defense responses to microbes and herbivores (Dixon, 2001). A subset of tryptophan (Trp)-derived secondary metabolites are specially produced in Cruciferous plants, including the model plant *Arabidopsis thaliana*, and play an important role in plant immunity (Buxdorf et al., 2013). Plants convert tryptophan (Trp) to indole-3-acetaldoxime (IAOx) by CYP79B2 and CYP79B3, and then to various bio-active compounds such as indole-glucosinolate-derivatives (IGs) and phytoalexin Camalexin. In particular, the myrosinase PENETRATION2 (PEN2) converts 4-methoxy indolyl-3-methyl glucosinolates (4MI3G) to IGs, and provides a critical step in fungal defense against various types of host-adapted and non-adapted pathogens and also for beneficial interactions with endophytes (Bednarek et al., 2009 and 2011; Hilbert et al., 2012; Hiruma et al., 2010;2016). In another branch from IAOx, Camalexin is synthesized by the cytochrome P450 monooxygenase PAD3. Camalexin-deficient *pad3* mutant plants display high susceptibility to various plant pathogens, such as *Alternaria brasicicola* (Zhou et al., 1999; Ferrari et al., 2003).

In this study, I aim to pursue the mechanisms by which plants in the field stay healthy even in the presence of potential pathogens without any signs of apparent immune activation or disease symptom. For this purpose, I pay attention to endophytic fungi in healthy Brassicaceae plants grown in the field. I have isolated and identified an as-yet-undocumented beneficial *Colletotrichum* fungus that protects plants from co-exiting pathogenic fungi. Importantly, this protection by CgE occurs without detectable plant growth retardation. Transcriptome and genetic analyses of the host plants have revealed several host factors (e.g., ET and tryptophan-derived metabolites) critical for the endophytic mode of CgE colonization in *A. thaliana* roots. Furthermore, together with collaborators, I have successfully discriminated fungal sequence reads derived from both beneficial and pathogenic fungi in co-inoculated roots to indeitify specific fungal genes that are activated during the fungus-fungus competition in roots. These results point to the importance of CgE-mediated direct suppression of pathogen growth in roots as a key step in host protection.

MATERIALS AND METHODS

Fungal isolation

Brassica family plants, including Chinese cabbage, Japanese mustard spinach, daikon radish, turnip, kale and cabbage were grown in an open field (Green laboratory, Nara Institute of Science and Technology) from November 2015 to January 2016. Mature healthy plants were harvested and washed in 15 minutes running tap water, followed by shaking in 1 minute of 70% Ethanol, then shaking in 1% Sodium Hypochlorite for 30 seconds, and finally washed by sterilized water for 3 times. Plant parts were left to dry and cut into small pieces approximate 3x3 mm, then place on BD Difco™ Potato Dextrose Agar (PDA, Becton, Dickinson and Company) in 60 mm petri dish. After a few days, pure cultures were obtained by picked up hyphal tip of visible colonies grown out of plant parts. Hypha was transferred to PDA and incubated at 25±1°C. Fungal isolates were also cryopreserved at -80°C in 25% Glycerol.

Molecular identification of endophytic fungi

Fungal mycelia were scraped from pure cultures grown on PDA medium for 2 weeks at 25±1°C in the dark. Mycelia were transferred to 0.3 ml PCR tubes with 100 µl of 10% Chelex® 100 Resin. Suspension was frozen by liquid nitrogen for 1 min and boiled at 95°C for 1 min in PCR machine. This procedure was repeated for 3 times. Then the suspension was finally boiled for 5 min. The suspension was vortex briefly and incubated at 55°C in PCR machine for 40 min and centrifuge at 12000 rpm for 1 min. DNA samples were collected from the supernatant of each tube. The Internal Transcribed Spacers (ITS) region on the ribosomal DNA was amplified with primer ITS1 and ITS4. The PCR was performed using KOD FX Neo® (Toyobo Life Science). PCR products were purified using FastGene Gel/PCR Extraction Kit (Nippon Genetics). These samples were used to a cycle sequencing with BigDye™ Terminator Cycle Sequencing reaction in PCR. The sequencing samples were then purified by ethanol/EDTA precipitation. The purified samples were sequenced using ABI 3100 sequencer. Sequences were assembled and edited by BioEdit, and then submitted to BLAST search.

Table 1: Primers used in this study

Gene/Region	Primer name	Nucleotide Sequence (5'-3')
AtACTIN2	ACT2F	ACCTTGCTGGACGTGACCTTACTGAT
	ACT2R	GTTGTCTCGTGGATTCCAGCAGCTT
ITS	ITS1F	CTTGGTCATTTAGAGGAAGTAA
	ITS4	TCCTCCGCTTATTGATATGC
CgETUB	CgETUB_F	ATGCGTGAAATCATCCACCTC
	CgETUB_R	GTAGTGCCCCTTGGCCCAGTTATT
CgEBOT2	CgEBOT2_F	ATGGCAACGCCCTTATGCCAA
	CgEBOT2_R	AGGCAATCGGTGACCAAATTG
GFP	GFP_F	GCGACGTAAACGGCCACAAGTT
	GFP_R	CATGGCGGACTTGAAGAAGT

Plant material and growth conditions

Arabidopsis thaliana accession Col-0 and the mutants were used for the study of plant interactions with endophytes and pathogens (Table 1). Seeds were surface sterilized with 70% ethanol for 30s and followed by 6% Sodium Hypochlorite plus 0.01% Triton X for 15 min. After three times wash in sterilized water, seeds were put in cold treatment at 4°C for 72 hours. The treated seeds were sown on the half strength MS media with 25 mM Sucrose. Plates were placed vertically in a plant growth chamber with a photoperiod of 12 h of light/ 12 h of darkness and temperature of 23±1 °C. For the inoculation assay, plants were transferred to the Pi-sufficient (625 μM KH_2PO_4) or Pi-deficient (50 μM KH_2PO_4) half-strength MS medium.

Table 2. *Arabidopsis thaliana* lines using in this study

Line name	Reference
Col-0	-
dde2 ein2 pad4 sid2	Tsuda et al., 2009
ein2 pad4 sid2	Tsuda et al., 2009
dde2 pad4 sid2	Tsuda et al., 2009
dde2 ein2 sid2	Tsuda et al., 2009
dde2	Malek et al., 2002
ein2-1	Alonso, 1999
ein3-1	Roman et al., 1995
pad4-1	Jirage et al., 1999
sid2-2	Wildermuth et al., 2002
cyp79B2 cyp79B3	Zhao, 2002
pad3	Zhou, 1999
pen2	Lipka et al., 2005

Fungal screening by plant and fungal co-cultures

Fungi with unique colony morphology and/or spore formation in our media were selected for the further assay. To observe their interactions with host, two agar plugs (7 mm diameter) of 10 day-old fungi were cut with a sterile cork-borer, then transferred to half strength MS media in 9 cm square plates. After 5 days, 7 day-old plants were placed to those media at 2 cm distance from the edge of fungal colonies. A sterile PDA plug was used as negative control. Plates were placed horizontally in a temperature control room with a photoperiod of 12 h of light/12 h of darkness and temperature of 23±1°C. Plant shoot fresh weight was measured after 21 days after infection.

Dual culture assay *in vitro*

Agar plugs of 10-day-old culture of CgP and CgE were transferred to PDA plates, each agar plug was placed 2.5 cm apart (edge-to-edge). Sterile paper disc with 5 μl of 10 mM of Hygromycin B were used as positive control. Agar plugs for non-cultured PDA plates and paper discs with 5 μl of sterile water were used as negative control. Colony formation and colony inhibition of fungi were observed at 10 days after inoculation by measuring the colony radius from center-to-edge.

Fungal inhibition assay by tryptophan-derived secondary metabolites

Agar plugs of 10-day-old culture of CgP and CgE were transferred to half strength MS media containing 100 μM tryptophan-mediated metabolites in 60 mm petri dish (Table 2). medium with Dimethyl Sulfoxide (DMSO) were used as negative control. Colony formation and colony inhibition of fungi were observed at 10 days after inoculation by measuring the colony radius from center-to-edge.

Table 3. List of tryptophan-mediated metabolites used in fungal inhibition assay

Metabolite name	Abbreviation
Camalexin	Cam
Indole-3-ylmethylamine	I3A
Indole-3-carbinol	I3C
4-hydroxyindole-3-carbonitrile	4OHICN

Plant-fungus interaction assay by plant and fungal co-cultures

Candidate fungus CgP and CgE were used for plant protection assay. The 7 days-old plants grown in half MS with sucrose were placed to half MS media without sucrose in 9 cm square plates. Spore suspensions of CgP, CgE and the mixed suspension (Table 3) were droped onto the plant root tips (10 μl each plant). The initial spore suspension of CgP and CgE in each treatment were adjusted to the same amount (500 spores/plant). Plates were placed horizontally in a temperature control room with a photoperiod of 12 h of light/ 12 h of darkness and temperature of 23±1 °C. Biomass of CgP as indicator of the root colonization rate were measured by qRT-PCR at 3 dpi. Plant shoot fresh weight and plant morphology were observed after 14 or 21days after infection.

Table 4: Mixture of spore suspension used in plant protection assay

	CgP (10^5 Spores/ml)	CgE (10^5 Spores/ml)	Sterile water
Control	0	0	1 ml
CgP	0.5 ml	0	0.5 ml
CgE	0	0.5 ml	0.5 ml
Co-inoculation	0.5 ml	0.5 ml	0

Quantitative Real-Time PCR (qPCR) Analyses of Gene Expression

Total RNAs were extracted using the Purelink™ RNA Purification solution (invitrogen), following by chloroform purification and alcohol precipitation. A 500-ng quantity of the RNAs was reversely transcribed to cDNA using Primescript™ reverse transcriptase (Takara). qPCR analyses were carried out using SYBR™ green (Applied biosystems) on Thermal Cycler Dice® Real Time System II (Takara). *ACTIN2* (At3g18780) or *CgETUB* (CGE14433) mRNA were used as an internal control, and the relative expression level of each gene was calculated by the $2^{\Delta\Delta_{Ct}}$ method (Livak & Schmittgen, 2001).

Fungal transformation via Agrobacterium-mediated transformation

The constitutively expressing GFP line of CgE and CgP were generated by Agrobacterium-mediated transformation using the binary plasmid pBin-GFP-hph (O'Connell et al., 2004) with the GFP gene under control of the constitutive GPDA promoter. Fungal transformants were selected by PDA containing 100 μM Hygromycin B.

Fluorescence Microscopy

Inoculated *A. Thaliana* roots at 1, 2, 3 and 7 dpi were visualized under fluorescent microscopy. The studies were performed on the confocal laser scanning microscope Olympus FV1000 with excitation at 488 nm for GFP or bright field and 560 nm for Propidium Iodide (PI) at 10x, 20x and 40x magnification. PI (10 mg/ml) was used for staining the cell wall of the roots by direct application on the slide.

Genome sequencing and assembly

Fungal DNA were extracted from fungal hyphae grown in liquid mathur's medium (Glucose 2.8 g/l, $MgSO_4 \cdot 7H_2O$ 1.2 g/l, KH_2PO_4 2.7 g/l and Mycological peptone LP0040 2.2 g/l) for 2 days. About 200 mg homogenized fungal hyphae were mixed with CTAB extraction buffer (2% cetyl trimethylammonium bromide, 1% polyvinyl pyrrolidone,100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA). The homogenates were then incubated at 60°C for 30 minutes and centrifuge for 5 minutes at 14,000 x g. Supernatants were transferred to new tubes and subjected to 5 μl of RNase solution A and incubated at 32°C for 10 minutes. The equal volume of chloroform/isoamyl alcohol (24:1) were added to the tube and centrifuged for 1 minutes at 14,000 x g. The aqueous upper phase was transferred to new tubes and DNA was precipitated by adding 0.7 volume cold isopropanol and incubate at -20°C for 10 minute then centrifuged for 10 minutes at 14,000 x g. Supernatants were decanted without disturbing the pellet and the pellet was subsequently washed with 500 μl cold 70% ethanol. The ethanol was the decanted and the pellet was dried. DNA samples were dissolved in 20 μl TE buffer (10 mM Tris, pH 8, 1 mM EDTA).

Genomic sequences of CgE and CgP were determined using PacBio single-molecule real-time sequencing and Illumina HiSeq for paired-end short reads. For PacBio sequencing, a SMRTbell library was constructed using the SMRTbell Express Template Kit (Pacific Biosciences, CA, USA) according to the manufacturer's protocol. The sequencing library was size-selected using the BluePippin system (Saga Science, MA, USA) with a minimum fragment length cutoff of 30 kbp. One SMRT cell (1M v2) was run on the PacBio Sequel System with Binding Kit2.0/Sequencing Kit2.1 and 600 min movies. In addition, genomic DNA was fragmented to an average size of 600 bp with the DNA Shearing System M220 (Covaris Inc., MA. USA). A paired-end library was constructed with a TruSeq DNA PCR-Free Library Prep kit (Illumina, CA, USA) and was size-selected on an agarose gel using a Zymoclean Large Fragment DNA Recovery Kit (Zymo Research, CA. USA). The final library was sequenced on the Illumina HiSeq 2500 sequencer with a read length of 250 bp.

The PacBio sequence reads were trimmed and assembled initially using Canu (version 1.6) (Koren et al., 2017), this initial assembly was polished to correct sequencing errors twice. The first polishing was performed using Arrow (version 2.2.1) (Chin et al. 2013) with PacBio long reads alignment from PBalign (version 0.3.1). The second polishing was performed using Pilon (version 1.22) (Walker et al. 2014) with Illumina short reads alignment from BWA (version 0.7.15) (Li and Durbin 2010).

Gene prediction and annotation

Protein-coding region identification and gene prediction were conducted through a combination of RNA-seq-based prediction, homology-based prediction, and de novo prediction methods. For RNA-seq-based prediction, RNA-seq reads were mapped to assembled genomes using HISAT (version 2.1.0) (Kim et al. 2015) with default parameters to identify exon regions and splice positions, and gene structure predictions were made by StringTie (version 1.3.4d) (Pertea et al. 2015) with default parameters. For homology-based prediction, protein sequences from fourteen species (*C. fructicola* strain Nara gc5, *C. gloeosporioides* strain cg14, *C. graminicola* strain M1 001, *C. higginsianum*, *C. orbicular*, *C. tofieldiae, C. chlorophyte, C. fioriniae, C. incanum, C. nymphaea, C. orchidophilum, C. salicis, C. simmondsii, C. sublineola*) were aligned to assembled genomes using Exonerate (version 2.2.0) (Slater and Birney, 2005). For ab initio gene prediction, GeneMark-ES (Version 4.33) and BRAKER (Version 2.1.0) were used (Ter-Hovhannisyan et al., 2008). GeneMark-ES was trained on its own genome sequence, and the resulting bam file from HiSat2 was used to train BRAKER. All gene models predicted from the above three approaches were combined by EvidenceModeler (version 2.2.0) into a nonredundant set of gene structures (Haas et al., 2008).

For functional annotation, the predicted gene sequences were searched with various databases such as, ntdb of NCBI and SwissProt, with a cut off E value of 1e-5. The predicted subcellular localizations were determined using SignalP (Nielsen et al., 1997) to identify N-terminal signal peptides and then TMHMM (Krogh et al., 2001) and Fungal BIG-PI (Eisenhaber et al., 2004) to exclude sequences with transmembrane domains and GPI-anchors. The sequences were then submitted to TargetP (Emanuelsson et al., 2000) analysis to identify predicted sequences for extracellular localization.

Orthologs among *Colletotrichum* species

We classified all the proteins from 16 *Colletotrichum* species into orthologous groups using Proteinortho (Lechner et al. 2011). This tool constructs groups on the basis of an all-against-all alignment of Blastp. The definition of an orthologous relationship between proteins was as follows: e value, ≤ 10 to 5; identity, ≥ 25%; and alignment coverage, ≥ 50% for both sequences.

Construction of a phylogenetic tree of concatenated protein-coding gene sequences

We determined orthologous groups of proteins from 16 *Colletotrichum* species (see the "Orthologs among *Colletotrichum* species" section) and extracted 4650 groups that had a one-to-one relationship across all species. For each group, multiple alignments were performed by MAFFT (Katoh and Standley 2013) and sites containing gaps ('-') or ambiguous characters ('X') were excluded. All alignments were concatenated and 94,749 amino acid sites were used for phylogenetic analysis. The phylogenetic tree was constructed with RAxML (version 8.2.12) (Stamatakis 2014). Here, we applied the JTT substitution matrix with a gamma model of rate heterogeneity (-m PROTGAMMAJTT) and the number of 1000 replicates for bootstrap analysis.

Transcriptome analysis

RNA samples were extracted from inoculated roots at 6 hour-post inoculation (6 hpi) and 3 days-post inoculation (3 dpi). Total RNA was extracted using NucleoSpin RNA Plant (Macherey-Nagel). RNA samples (1μg each) were then sent to Macrogen for library preparation and subsequent sequencing. The generated libraries were sequenced by HiSeq-illumina (approximately 20 million reads per sample, paired-end). For plant transcriptome analysis, Tophat2 with default setting (Kim et al., 2015) was used for sequence mapping to the reference *A. thaliana* genome (TAIR10). The generated BAM files from Tophat2 platform are

used to analysis differentially expressed genes by cuffdiff with default setting (Trapnell et al., 2012). The following statistical analysis and visualization of data were conducted by R Bioconductor packages, such as Cummerbund and ggplot2 (Wickham, 2016; Goff et al., 2019).

RNA-seq read sets obtained from CgE, CgP, and co-inoculation samples were subjected to adapter removal, and to quality filtering using Platanus_trim (version 1.0.7) with default parameters. The trimmed reads were classified using two sequential rounds of mapping. First, the trimmed reads were mapped to the Arabidopsis genome using HISAT (version 2.1.0) (Kim et al., 2015) with default parameters. Reads that were mapped onto the Arabidopsis genome were classified as originating from Arabidopsis. Next, we performed the second classification by mapping the reads that remained unmapped in the first classification onto CgE and CgP genomes. Reads that uniquely mapped to either CgE or CgP genomes were classified as originating from CgE or CgP, respectively. In addition, reads that could not be mapped to both genomes were classified as unmapped reads. Finally, reads that mapped to both genomes during the second step were further classified. The number of mismatches in the alignment reads were identified by the "XM" flag in SAM output files, and then compared the number of mismatches in the alignment against CgE and CgP genomes. Based on comparison results, reads were classified into CgE, CgP, and classified reads. The resulting CgE-RNA-seq derived CgE reads (CgE-CgE reads), CgP-RNA-seq derived CgP reads (CgP-CgP reads), co-inoculation-RNA-seq derived CgE reads (co-inoc-CgE reads), and co-inoculation-RNA-seq derived CgP reads (co-inoc-CgP reads) were used for differential expression analysis.

CgE-CgE reads and co-inoc-CgE reads were mapped to annotated genome of CgE, and CgP-CgP reads and co-inoc-CgP reads were mapped to the annotated genome of CgP using HISAT2 (version 2.1.0). The reads of each sample were used for transcript assembly and quantification of each transcript using StringTie (version 1.3.4d) (Pertea et al. 2015) and Ballgown R package (Frazee et al. 2015). To identify differential expressed genes (DEGs), the edgeR package (Robinson et al. 2010) was used to compute the p-value and fold change. The p-value was used to identify genes expressed differentially between the paired datasets. Significantly DGEs were identified using a FDR (false discovery rate) threshold of ≤ 0.05 and a minimum two-fold change.

RESULTS

Isolation and screening of endophytic fungus candidates from Brassica plants in fields

A total of 116 isolates for fungal endophyte candidates were isolated from roots and/or leaves of *Brassica* spp. plants after surface sterilization. Among these fungi, ten isolates were chosen for further analysis based on their suitable morphological and growth characteristics for inoculation assay on plants.

I found that when separately inoculated with these fungi for 21 days, Arabidopsis plants display from an increase to a decrease in shoot fresh weight (SFW), which can be defined from plant growth promotion to inhibition (Figure 1 and 2). In particular, inoculation with the fungal isolates (E35, E41, E62 and E66) under nutrient-sufficient conditions increased SFW by 17%, 56%, 22% and 22%, respectively, relative to that of the non-inoculated samples. The maximum SFW promotion was observed when inoculated with E41 (56%). In contrast, some isolates (E6, E40, E59, E67 and E74) significantly decreased plant SFW by 43%, 28%, 35%, 12% and 44%, respectively, relative to that of non-inoculated samples. The maximum SFW reduction was observed when inoculated with E74 (44%). These results suggest that healthy plants in nature not only interact with endophytic but also potentially pathogenic fungi.

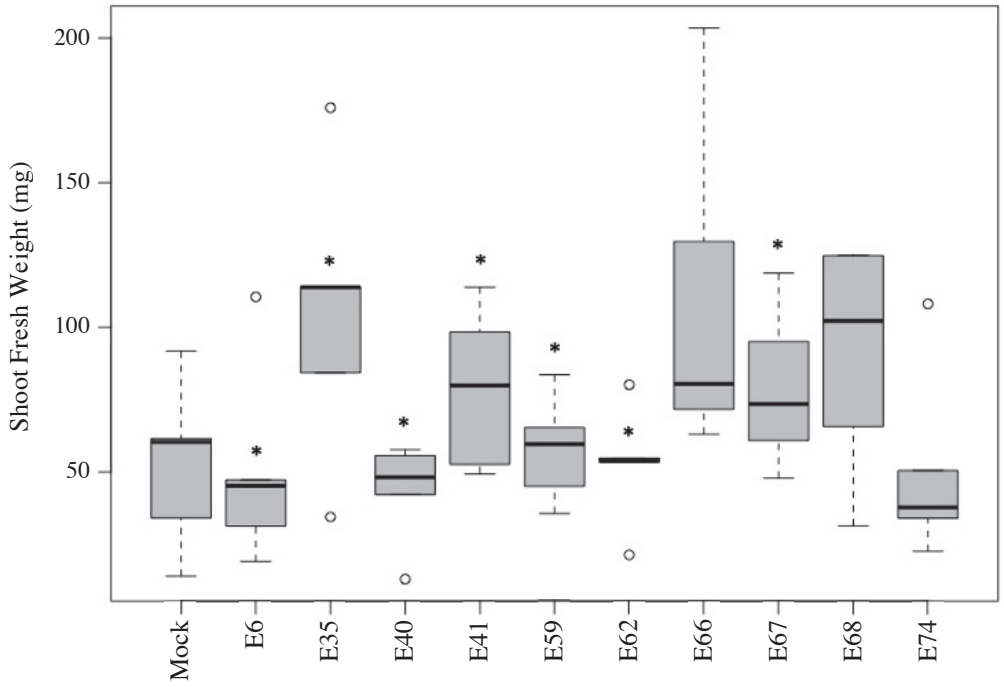


Figure 1. Shoot fresh weight of *Arabidopsis* when inoculated with fungal isolates determined at 21 dpi**.** Ten shoots were subjected to the determination per sample and experiment. Asterisks indicate significant differences in the means between mock and fungal inoculated plants**.** (two-tailed t-test, $p<0.05$)

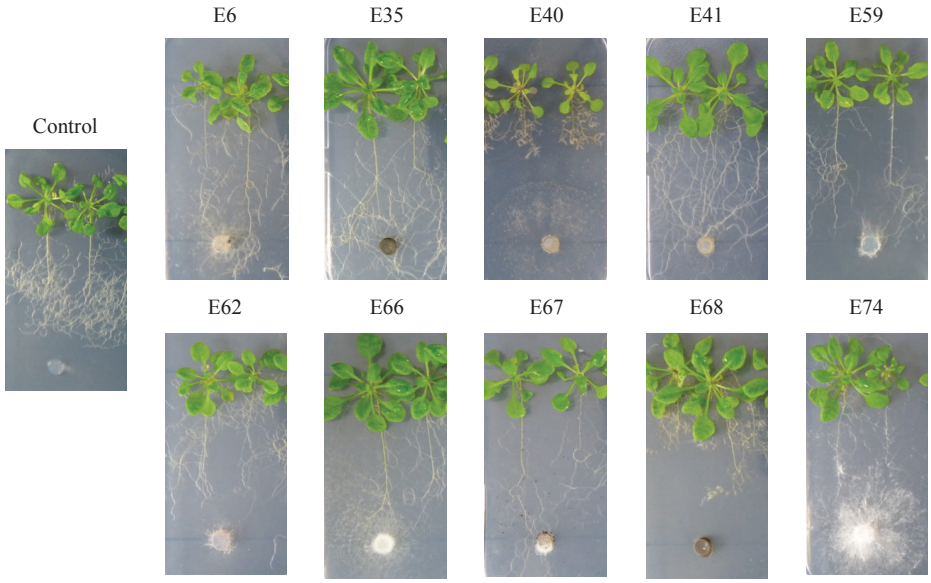
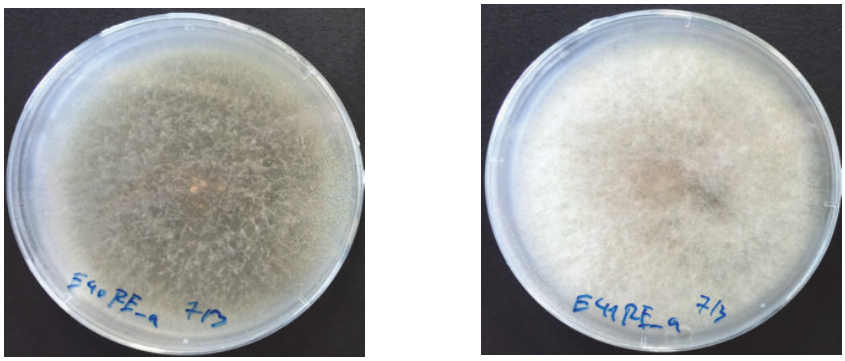


Figure 2. Morphology of plants when inoculated with fungal isolates at 21 dpi on 1/2 x MS agar media.

Interestingly, despite their opposite effects on plant growth, pathogenic fungus E40 and endophytic fungus E41 showed highly similar colony morphology characteristic of *Colletotrichum* genus on potato dextrose agar media (Figure 3)(Perfect et al., 1999; cannon et al., 2003; Weir et al., 2012). To validate whether these fungi belong to *Colletotrichum* genus, I determined Internal Transcribe Spacer (ITS) sequences from the two fungi. ITS sequence analysis predicted that these fungi were closely related with each other and both belong to *Colletotrichum gloeosporioides* (Table 5). Importantly, these fungi were isolated from the same part of healthy radish (leaves) at the same timing. Co-existing of these two fungi in healthy plants implied a possibility that endophytic fungus E41 (Endophytic *Colletotrichum gloeosporioides*: CgE) has a role in host plant protection against pathogenic fungus E40 (Pathogenic *Colletotrichum gloeosporioides*: CgP)



Colletotrichum gloeosporioides *Colletotrichum gloeosporioides*

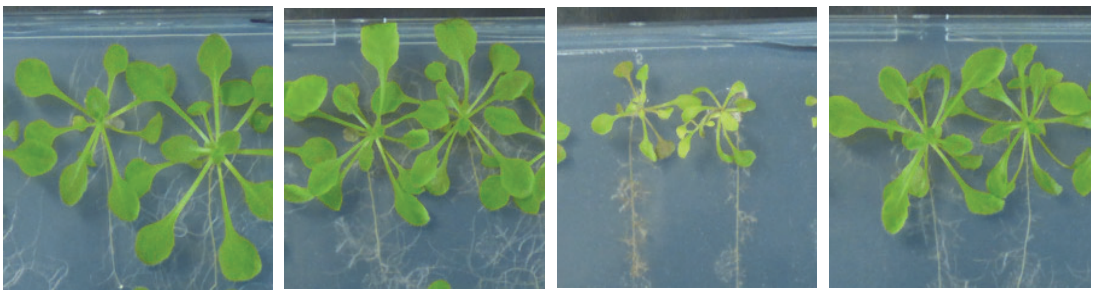
Figure 3. Colony morphology of pathogenic *Colletotrichum spp*. (E40) and endophytic *Colletotrichum spp*. (E41) isolated from the healthy radish leaves grown during autumn 2015. Fungi were cultured in potato dextrose agar medium at 25 °C for 10 days.

Table 5. Closest match of fungal isolate ITS sequence inferred from BLASTn search in GenBank.

Isolate	Fungal acronyms	Original host	Organ source	BLASTn closest match	%ITS Similarity
E40	CgP	radish	leaf	*Colletotrichum gloeosporioides* Isolate WY2-1 (KT218694.1)	97%
E41	CgE	radish	leaf	*Colletotrichum gloeosporioides Strain DZ-1-3-2* (KT192230.1)	99%

Endophytic *Colletotrichum gloeosporioides* (CgE) protects plants from pathogenic *Colletotrichum gloeosporioides* (CgP)

To test whether CgE has a role in host protection against a pathogen, spores of CgP and CgE were co-inoculated with Arabidopsis roots under nutrient sufficient conditions. Consistent with the results above, inoculation of CgP severely inhibited plant growth at 21 dpi, as shown with shoot fresh weight (SFW) (Figure 4). In contrast, visible disease symptoms and growth retardation were not observed when inoculated with CgE. Importantly, plants co-inoculated with CgP and CgE showed significantly less disease symptoms (Figure 4 and 5) compared to that with CgP alone. These results indicate that CgE protects *A. thaliana* plants from plant growth inhibition caused by pathogenic CgP.



Mock CgE CgP CgE+CgP

Figure 4. Morphology of plants treated either with water (mock), endophytic *Colletotrichum* spp.(CgE), pathogenic *Colletotrichum* spp. (CgP) and co-inoculation of CgP with CgE, respectively at 21 dpi on 1/2MS agar media.

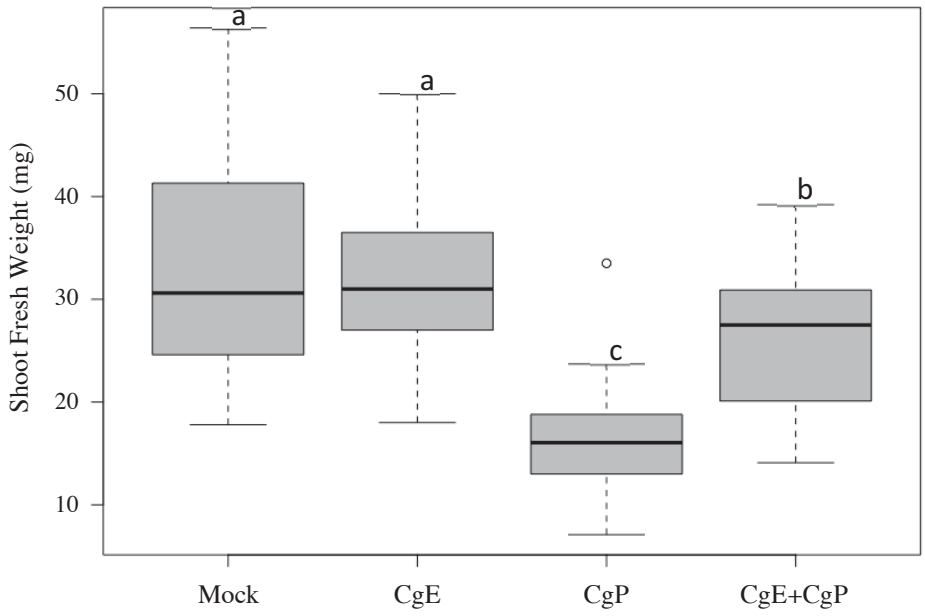


Figure 5. Shoot fresh weight of *Arabidopsis* in a co-inoculation assay at 21 dpi. Each sample had 10 shoots tested per experiment. The boxplot shows combined data from three independent experiments. Different letters indicate significantly different statistical groups (Tukey-HSD, $p < 0.05$).

CgE does not inhibit CgP in the absence of the host plants

Fungal growth inhibition mechanisms can be largely classified into two ways, direct and indirect effects. Direct effects include nutrient competition and production of antifungal compounds. Direct effects by endophytes have been often observed in the absence of the host organisms. In contrast, indirect effects typically require host plants to activate defense responses that prevent infection of pathogens. To test first whether CgE directly inhibits CgP even *in vitro* in the absence of plants, the two fungi were co-incubated in culture. Antibiotic Hygromycin B was used as a positive inhibitor, which effectively suppressed CgP colony growth (Figure 6). In contrast, when CgP and CgE were co-cultured on the same plates, CgP and CgE grew similarly to each other, indicating that CgE does not inhibit CgP growth in culture (Figure 6). Hence, plant protection by CgE may require its presence in the host.

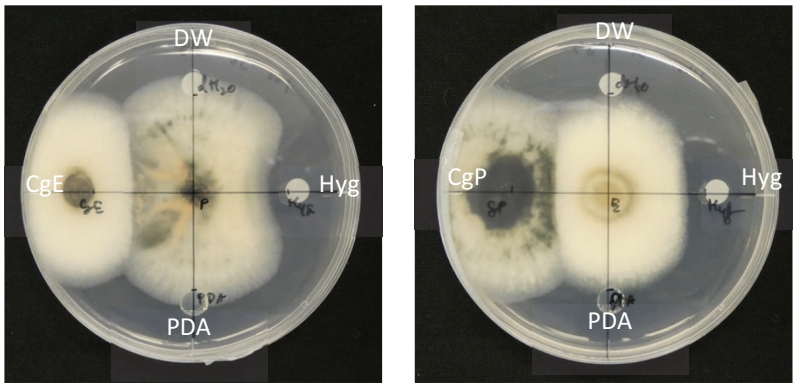


Figure 6. Colony morphology of CgP and CgE at 7 dpi on Potato Dextrose Agar (PDA) media. Fungal colonies were placed besides colonies of the other fungus, Potato Dextrose Agar plug, and filter paper disc contained water or Hygormycin B. In contrast to Hygormycin B, which clearly inhibited CgP colony expansion, CgE did not significantly inhibit CgP growth.

CgE suppresses CgP colonization in roots at an initial stage

To test whether CgE restricts CgP growth *in planta*, I assessed CgP colonization (fungal amounts) in roots when co-inoculated with and without CgE. To distinguish these two related fungi in roots, we used pathogenic *Colletotrichum gloeosporioides* (CgP-GFP) constitutively expressing green fluorescence protein (GFP). On co-inoculation of CgP-GFP with wild type CgE, I quantifed CgP colonization rate by assessing GFP signal or *GFP* expression. As CgP spores germinated equally either in absence or presence of CgE (at 6 hpi, data not shown), it is likely that CgE does not inhibit CgP spore germination (Figure 8). In contrast, CgP colonization at 3 dpi was reduced when co-inoculated with CgE (Figure 7). Microscopic observation also detected less dens hyphal network of CgP-GFP in plant roots in the presence of CgE than in its absence at 3 dpi. These results imply that CgE restricts hyphal growth of CgP at an early stage of CgP colonization in roots, and which probably occurs after CgP spore germination. In addition, CgP formed new generation of spores in the absence of CgE but not in its presence (Figure 8). These results suggest that co-colonization of CgE and CgP suppresses colonization of CgP in Arabidopsis roots to inhibit CgP reproduction.

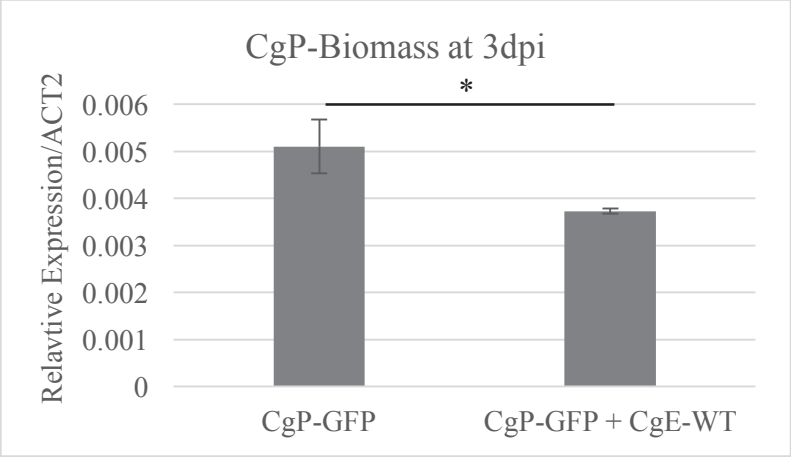


Figure 7. The GFP expression levels were determined as a proxy for fungal biomass by qRT-PCR at 3 dpi. Each sample had 20-25 roots tested per experiment. Data were obtained in three independent experiments. Bars represent means and standard errors of data collected in all independent experiments. (*T-test, $p<0.05$)

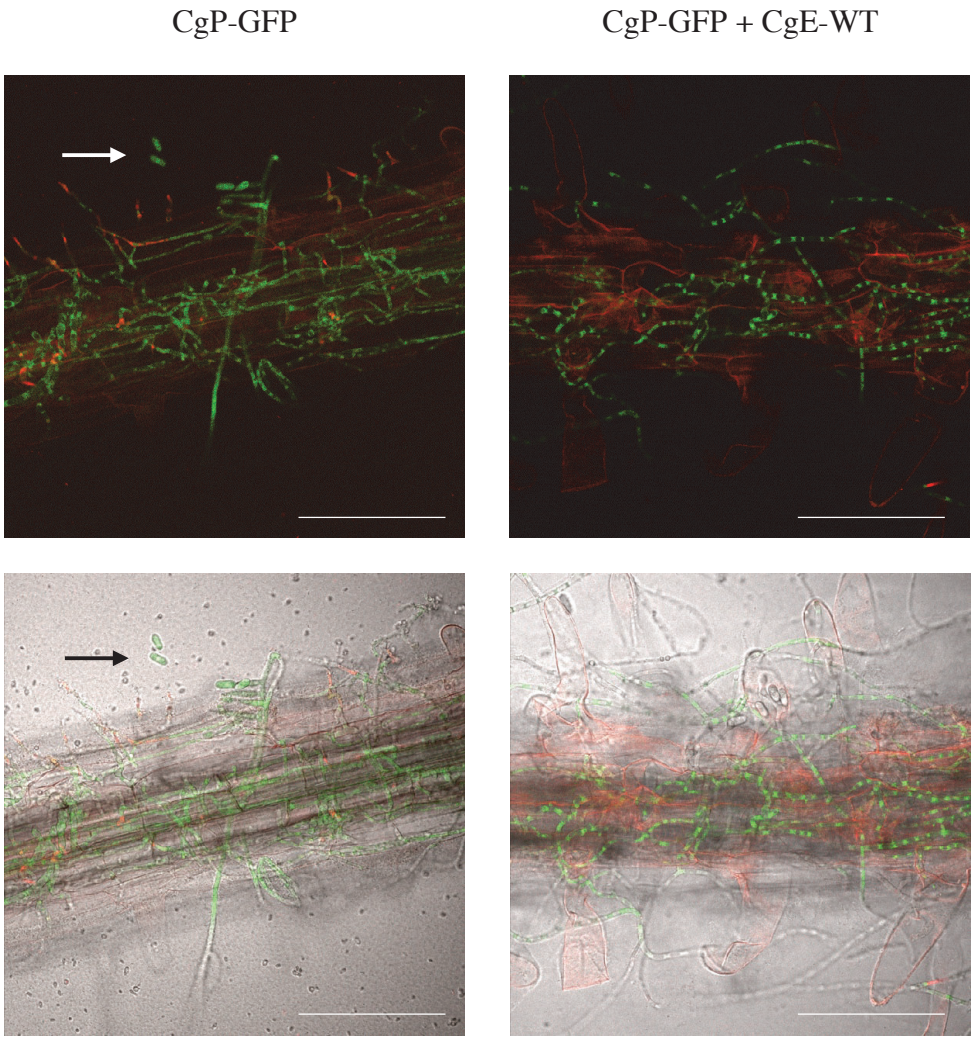


Figure 8. Confocal microscope images of pathogenic *Colletotrichum gloeosporioides* expressing cytoplasmic GFP (green) and *A. thaliana* with stained with Propidium iodide (red). Hyphal network of CgP-GFP colonized around the root (left panel) and co-colonized with CgE-WT (Right panel). Arrows indicate the newly generated spores of CgP-GFP in the *A. thaliana* root at 3 dpi. Scale bar, 100 μm.

CgE also protects plants from pathogenic *Colletotrichum incanum* (Ci)

To investigate whether CgE can protect plant from other pathogens, I inoculated CgE together with a pathogenic *Colletotrichum* spp., *Colletotrichum incanum* (Ci) (Sato et al., 2005; Hiruma et al., 2016; Hacquard et al., 2016), which is more distantly related to CgE as compared to CgP. CgE also relieved disease symptoms caused by Ci when co-inoculated, compared with when Ci alone was inoculated, suggesting that CgE also protects plants from Ci pathogenesis (Figure 9 and 10). However, the plant protection by CgE against Ci attack was not so robust as that against CgP (Figure 9 and 10). Therefore, protection ability of CgE may depend on a phylogenetic close relationship with pathogens.

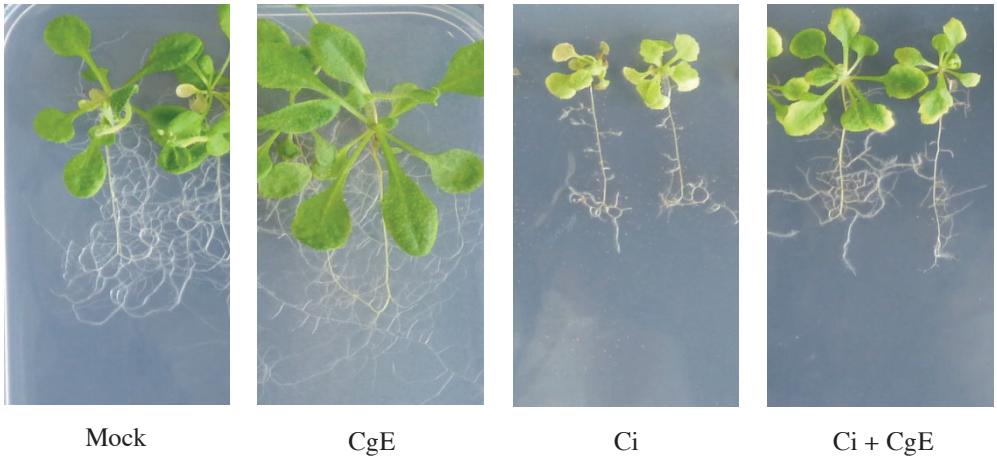


Figure 9. Morphology of plants treated with water, pathogenic *Colletotrichum incanum (Ci)*, co-inoculation of pathogenic *Colletotrichum incanum (Ci)* with endophytic *Colletotrichum* spp. (CgE), and endophytic *Colletotrichum* spp. (CgE), respectively at 21 dpi on 1/2MS agar media.

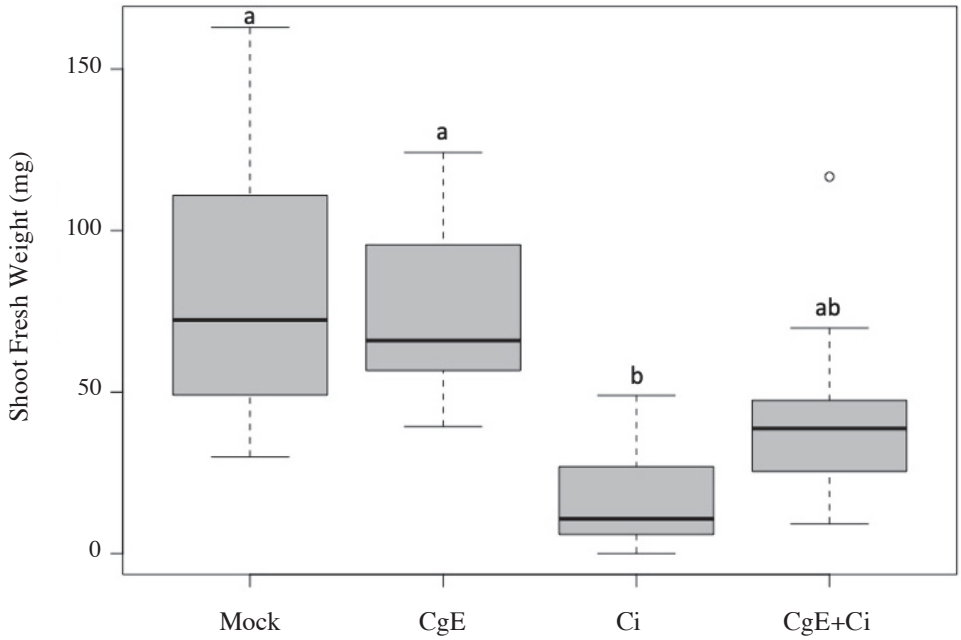


Figure 10. Shoot fresh weight of *Arabidopsis* from co-inoculation of pathogenic *Colletotrichum incanum (Ci)* with endophytic *Colletotrichum* spp. (CgE) assay at 21 dpi**.** The boxplot shows combined data from three independent experiments. Different letters indicate significantly different statistical groups (Tukey-HSD, $p < 0.05$)

Plant ethylene responses are required for the endophytic lifestyle and protection ability of CgE

ET, JA, SA are major plant defense-related hormones that regulate plant interactions with both pathogenic and endophytic fungi (Robert-Seilaniantz et al., 2011). To investigate whether these pathways influence the life-styles of CgE and plant protection mediated by CgE in roots, I first tested whether the beneficial interaction with CgE is altered in *ein2 sid2 dde2 pad4* quadruple mutant plants in which all three major phytohormone-related defense signaling pathways are simultaneously disrupted (Tsuda et al., 2009). I found that CgE-mediated plant protection is totally compromised in the mutant lines. Unlike the wild-type plants, CgE even strongly inhibited plant growth in the quadruple mutant. (Figure 11)

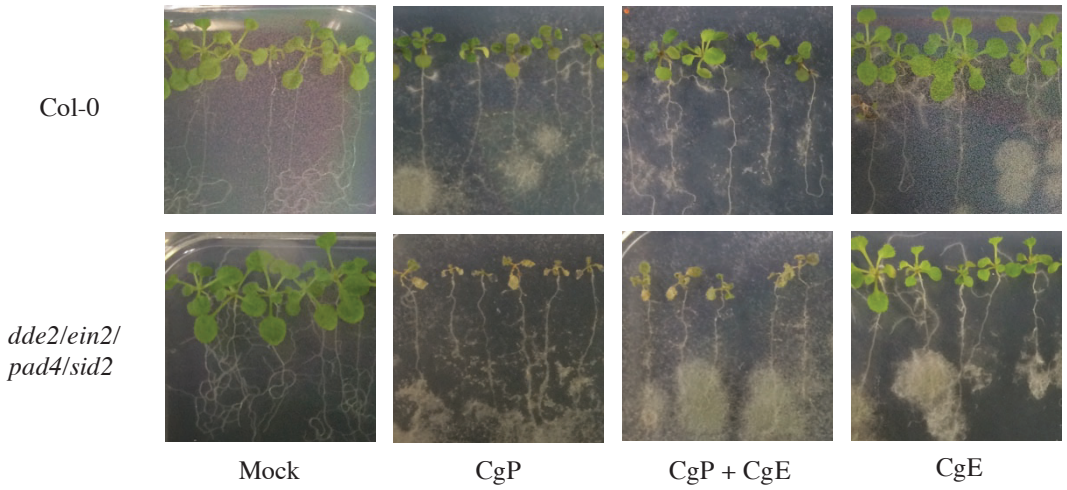


Figure 11. Morphology of plant treated with water, pathogenic *Colletotrichum* spp. (CgP), co-inoculation of pathogenic *Colletotrichum* spp. with endophytic *Colletotrichum* spp. (CgP+CgE), endophytic *Colletotrichum* spp. (CgE), respectively at 21 dpi on 1/2MS agar media.

To determine critical hormone pathway(s) for the endophytic behavior and/or plant protection ability of CgE, three triple mutants (*ein2*/*pad4*/*sid2*, *dde2*/*pad4*/*sid2* and *dde2*/*ein2*/*sid2*) were further tested. In these mutants, only one of the three hormone pathways is retained. Interestingly, when only ET signaling is present (*dde2*/*pad4*/*sid2*), plants survived after the co-inoculation with CgP and CgE (Figure 12 and 13). In contrast, the mutants, which maintain JA (*ein2*/*pad4*/*sid2*) and SA (*dde2*/*ein2*/*sid2)* but lack ET signaling, cannot survive in either fungal inoculation (Figure 12 and 13). These data suggest a role for ET in the establishment and/or maintenance of endophytic behavior and plant protecting function of CgE.

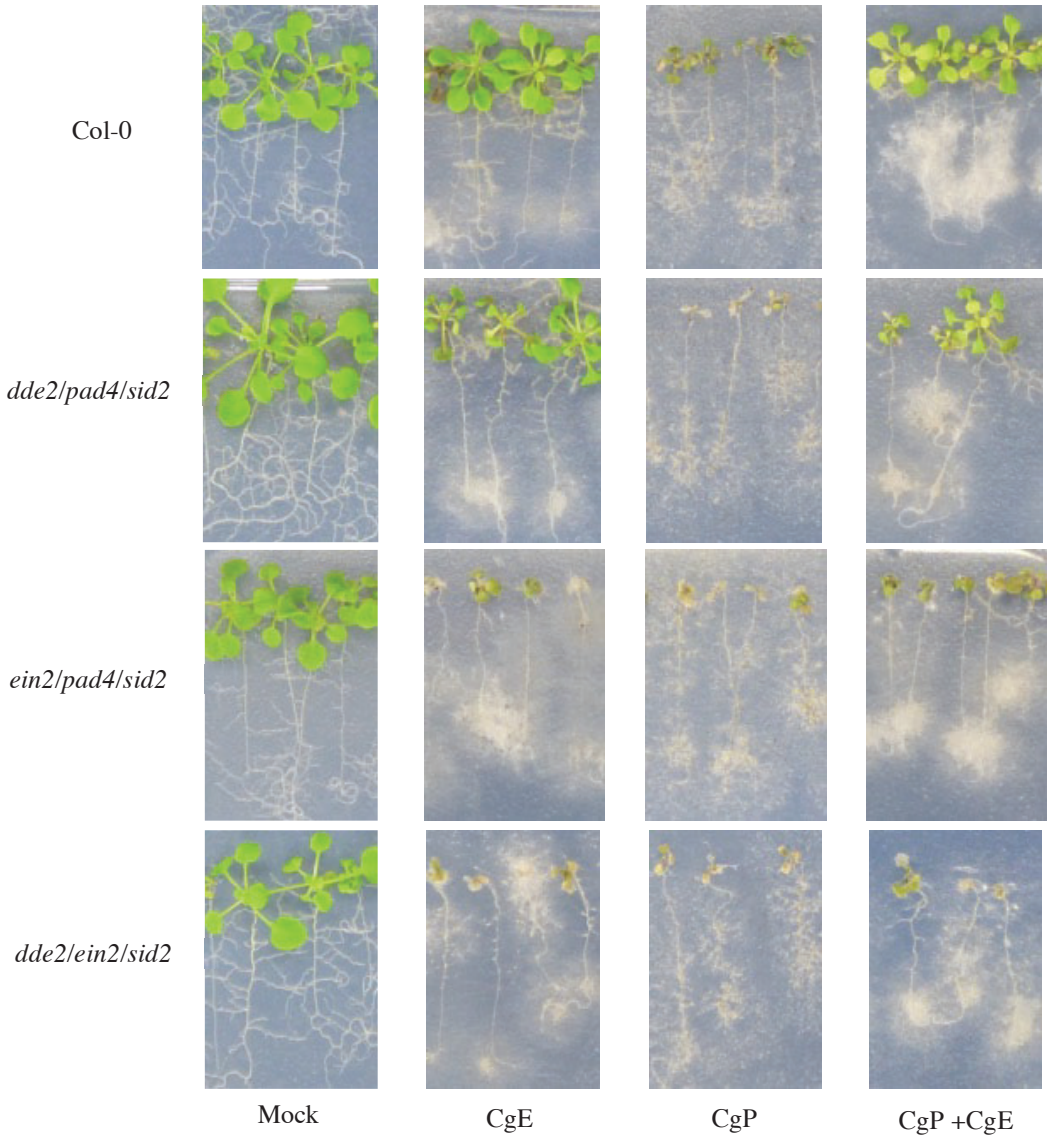


Figure 12. Morphology of triple mutants (*ein2*/*pad4*/*sid2*, *dde2*/*pad4*/*sid2* and *dde2*/*ein2*/*sid2*) compared to the Col-0 wild type 14 dpi with the indicated fungi on 1/2 x MS agar media.

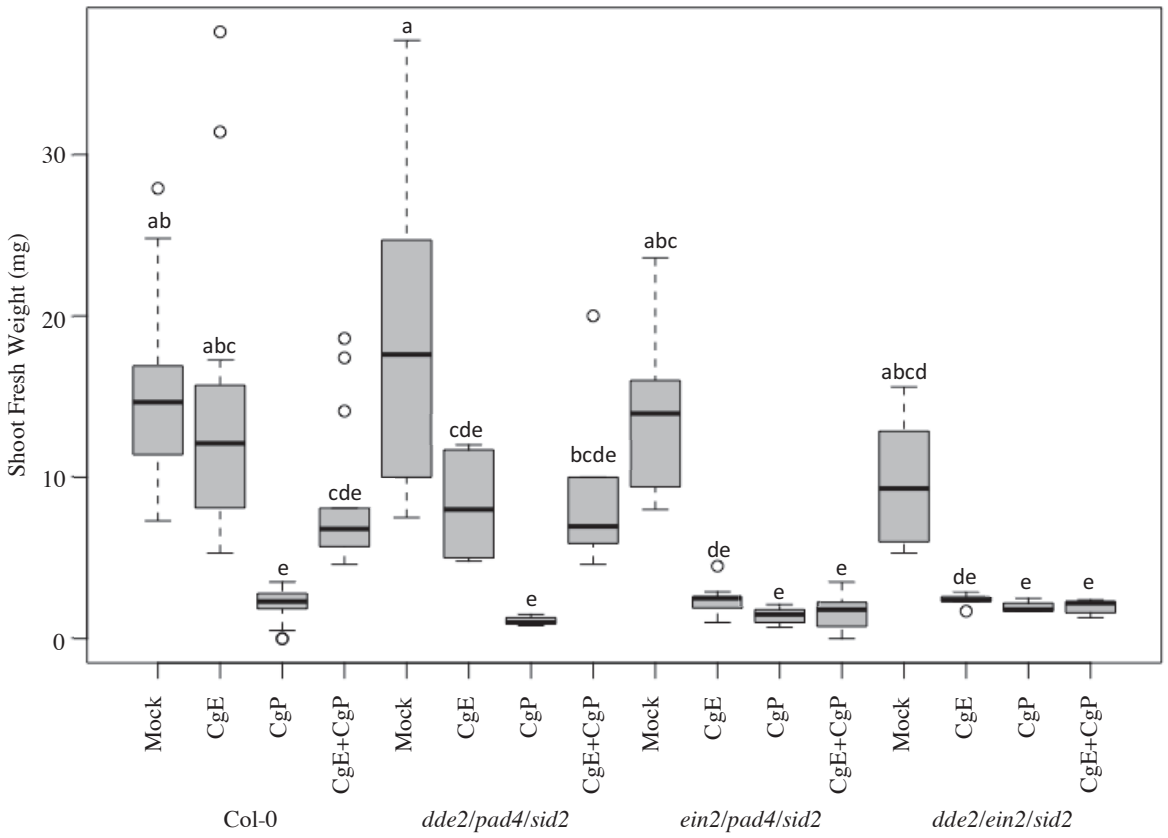


Figure 13. Shoot fresh weight of *Arabidopsis* triple mutants 14 dpi with the indicated fungi**.** The boxplot shows combined data from three independent experiments. Different letters indicate significantly different statistical groups (Tukey-HSD, $p < 0.05$).

To confirm the requirement of the host ET signaling pathway, an ET signaling pathway mutant (*ein2-1*) was tested. The mutant plants were susceptible to CgE and were not protected by CgE (Figure 14 and 15). In addition, I further analyzed another ET hyposensitive mutant, *ein3-1*. EIN3 identifies one of the master transcription factors for the ET responsive genes, which works downstream of EIN2 (An et al., 2010). *ein3-1* plants were also susceptible to CgE (Figure 14 and 15). These results indicate that CgE requires the host ET signaling to establish/maintain its endophytic (non-pathogenic) interaction with the host.

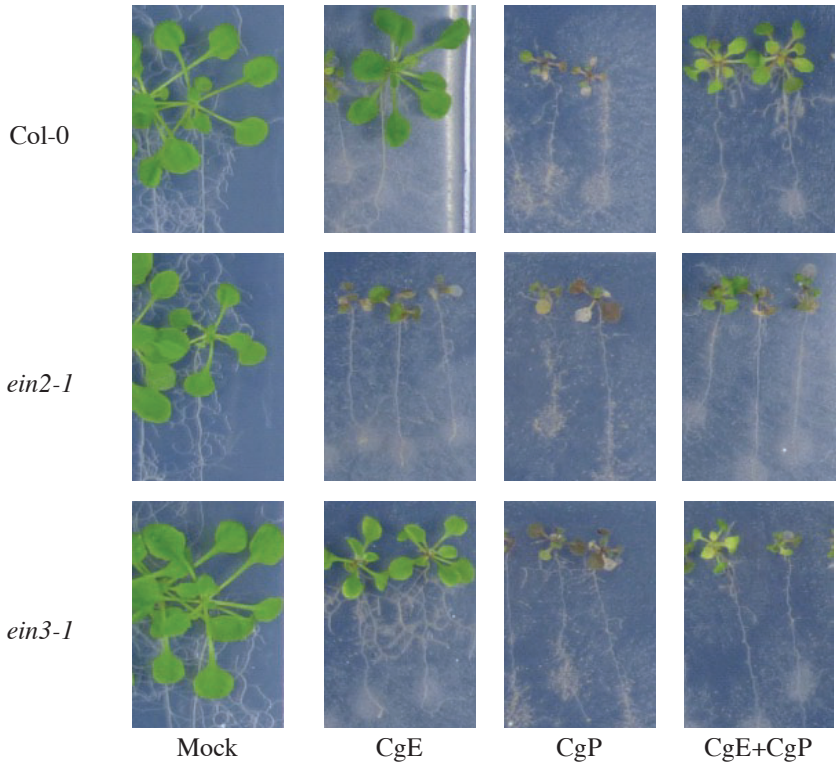


Figure 14. Morphology of *ein2-1* and *ein3-1* mutant plants 14 dpi with the indicated fungi on 1/2MS agar media.

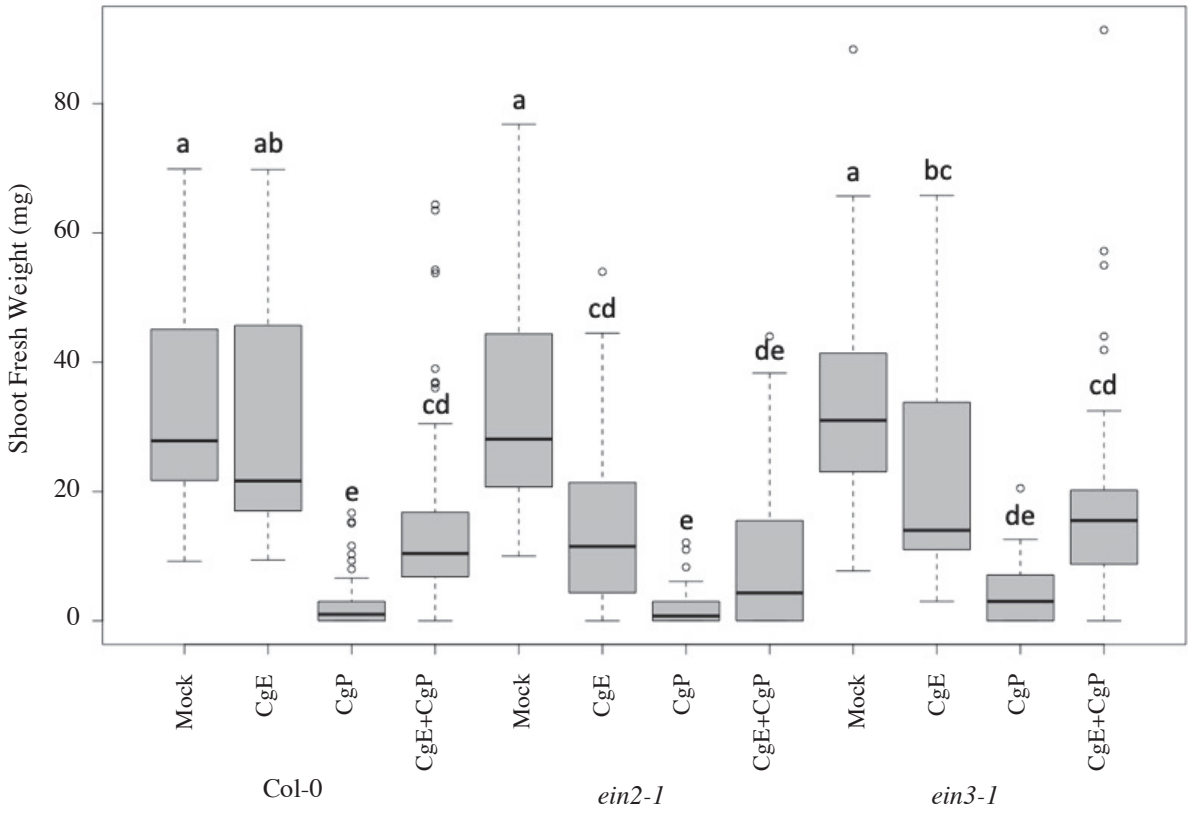


Figure 15. Shoot fresh weight of *Arabidopsis* triple mutants 14 dpi with the indicated fungi**.** Each sample had 20 shoots tested per experiment. The boxplot shows combined data from three independent experiments. Different letters indicate significantly different statistical groups (Tukey-HSD, $p < 0.05$).

Tryptophan-derived metabolites are required for endophytic lifestyle and plant protection ability of CgE

Tryprophan (Trp)-derived secondary metabolites in Arabidopsis regulate plant interactions with both pathogenic and endophytic fungi (Ferrari et al., 2003; Bednarek et al., 2009; Hiruma et al., 2016). I next investigated a possible role for this metabolic pathway of the host in endophytic life-styles and plant protection ability of CgE. I tested their possible alterations in the *cyp79B2 cyp79B3* plants that lack the cytochrome P450-mediated conversion of Trp to indole-3-acetaldoxime (IAOx), the first intermediate in this pathway (Figure 16). The mutant plants were more susceptible to CgP as compared to the Col-0 wild-type plants, as evident with discernible disease symptoms and the lack of protection from CgP (Figure 17 and 18).

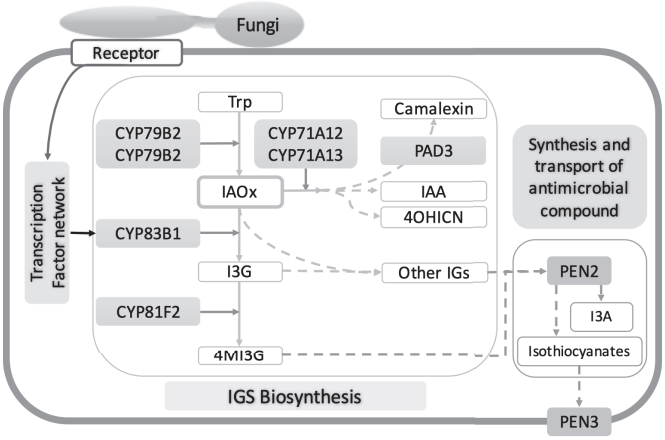


Figure 16. Trp-derived metabolic pathways in *A. thaliana*.

Furthermore, I tested two mutants for the components working downstream of CYB79B2 and CYP79B3, namely *pen2* and *pad3* mutant plants (Figure 16). The *PENETRATION2* (*PEN2*) gene encodes atypical myrosinase, which is required for pre-invasion resistance against pathogenic fungi and also for beneficial interactions with endophytic *C. tofieldiae* (Lipka et al., 2005; Peskan-Berghöfer et al., 2015; Hiruma et al., 2016). The *PHYTOALEXIN DEFICIENT3* (*PAD3*) gene encodes the cytochrome P450 CYP71B15, which catalyzes the conversion of dihydrocamalexic acid to camalexin (Zhou et al, 1999). These mutants were more susceptible to CgP than Col-0 was. Moreover, these mutants were also susceptible to CgE, as evident with the disease symptom. Consistent with these observations, CgE failed to protect plants from CgP virulence (Figure 17 and 18), suggesting that CgE became pathogenic and failed to protect plants from CgP when Trp-derived metabolites production is disrupted.

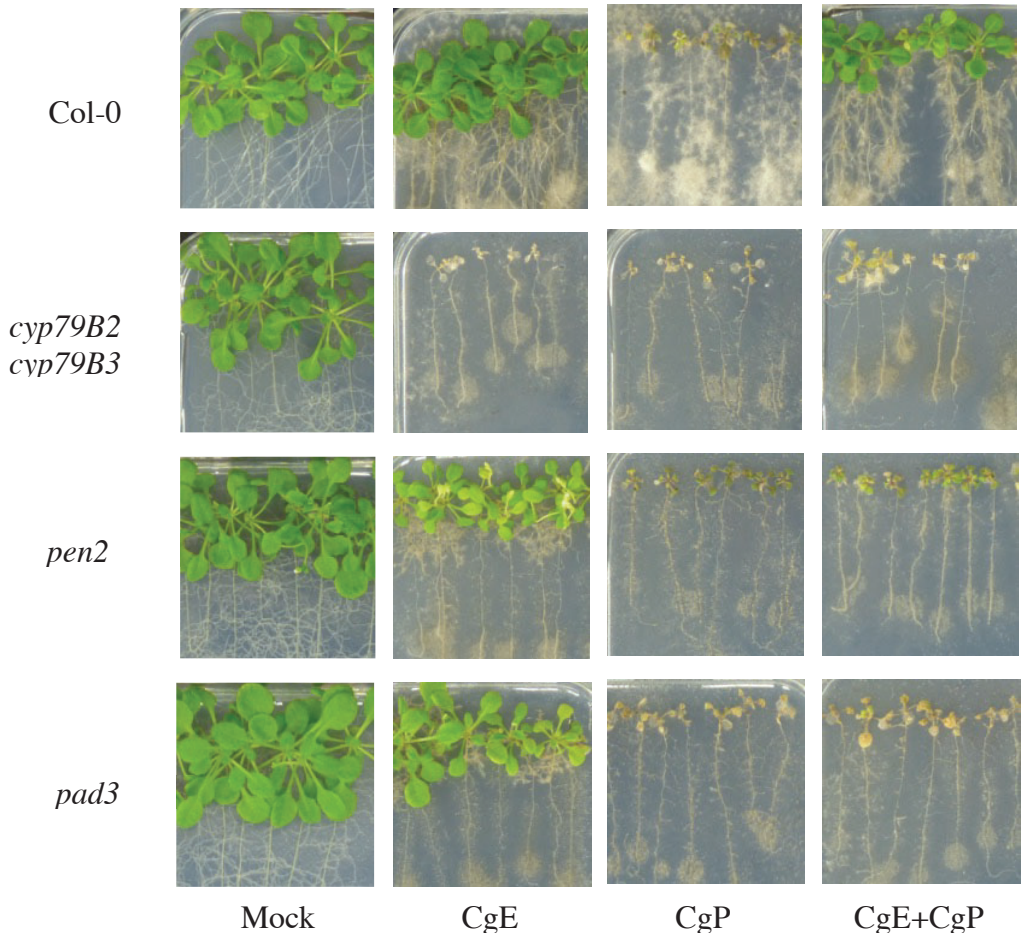


Figure 17. Morphology of trp-pathway mutant plants 14 dpi with the indicated fungi on 1/2 x MS agar media.

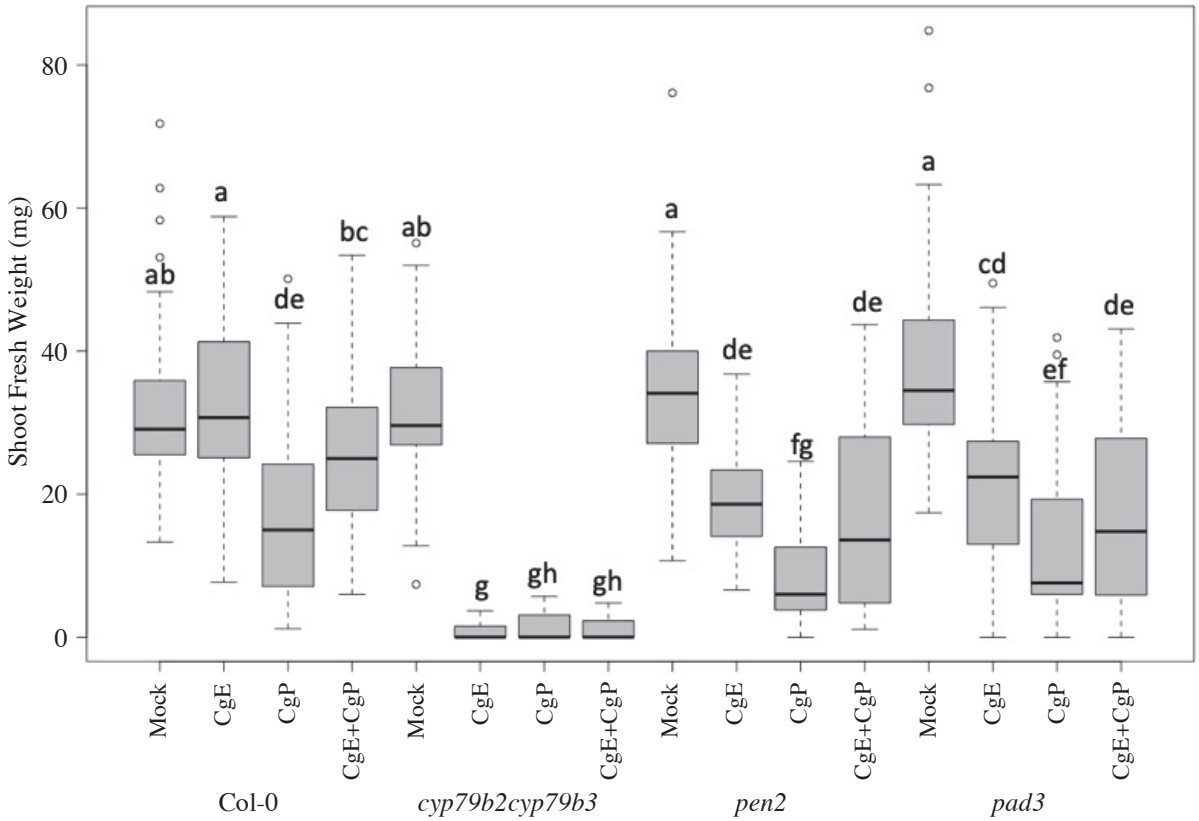


Figure 18. Shoot fresh weight of *Arabidopsis* mutants 14 dpi with the indicated fungi**.** The boxplot shows combined data from three independent experiments. Different letters indicate significantly different statistical groups (Tukey-HSD, $p < 0.05$).

In addition, I further verified the importance of Trp-derived metabolites in the control of fungal growth by assessing fungal growth in the media containing three different Trp-derived metabolites. In the presence of camalexin and Indole-3-carbinol (I3C), culture growth of CgE and CgP was suppressed as determined with colony diameter. In contrast, indole-3-ylmethylamine (I3A) and 4-hydroxyindole-3-carbonitrile (4OHICN) did not suppress CgE or CgP growth. Combined with the results obtained in *A. thaliana* mutants defective in Trp-derived metabolites, these results suggest that specific types of Trp-derived metabolites have direct inhibitory effects on fungal growth, which seem to be required to keep CgE as an endophyte (Figure 19 and 20). Interestingly, CgE showed higher tolerance toward camalexin and I3C than CgP did, implying that CgE is better adapted and tolerant to Arabidopsis roots, where high concentrations of anti-fungal Trp-derived metabolites are produced in response to fungal challenge.

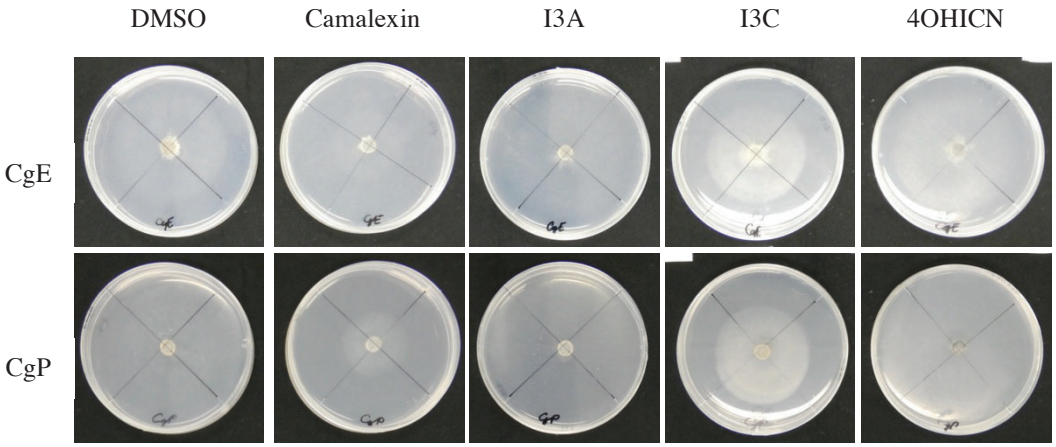


Figure 19. Morphology of 8 day-old CgE and CgP colonies cultured on 1/2 x MS agar media supplemented with Trp-derived secondary metabolites, Camalexin (Cam), Indole-3-ylmethylamine (I3A), Indole-3-carbinol (I3C) and 4-hydroxyindole-3-carbonitrile (4OHICN).

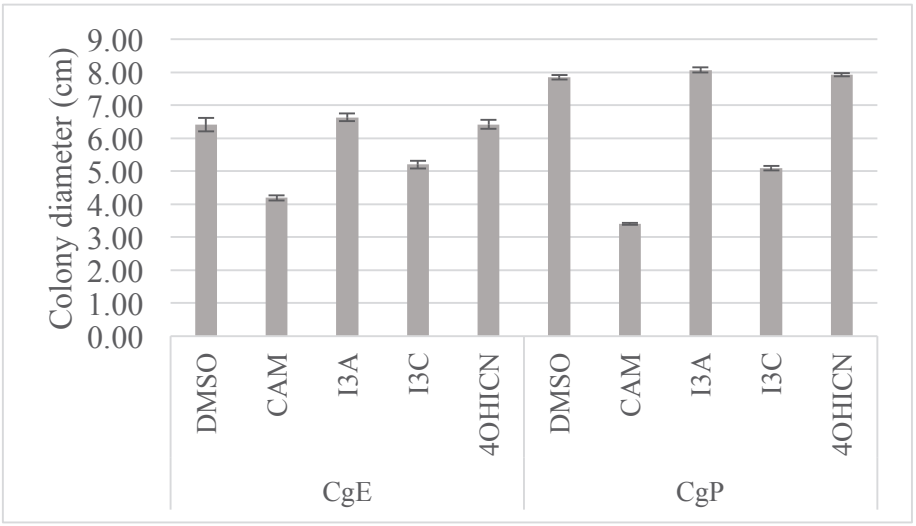


Figure 20. Colony diameter of 8 day-old CgE and CgP colonies cultured on 1/2 xMS agar media supplemented with Trp-derived secondary metabolites.

Host-side RNA sequencing analysis during fungal colonization in roots

To compare the host transcriptional dynamics during pathogenic and endophytic colonization in roots, I conducted RNA-sequencing analysis for mock-, CgE-, CgP-, and co-inoculated samples 6 h and 3 days after inoculation. To uncover the relationship between their transcriptome, we first conducted multidimensional scaling (MDS) analysis (Figure 21). At 6 hpi I did not detect clear separation among all samples, suggesting that extensive host transcriptome reprograming does not occur at this time point after fungal inoculation. In contrast, at 3 dpi, I detected a clear separation between mock-treated and fungus-inoculated samples. I also detected a separation between CgE-inoculated and CgP-inoculated samples, suggesting that the two fungal isolates induce differential transcriptome reprograming at this stage. Interestingly, co-inoculated samples were closely related to that of the CgE-inoculated samples rather than CgP-inoculated samples. Considering the fact that CgP growth in roots is suppressed at this time (Figure 21 and 22), this suggests that CgE is a major driver for the transcriptome reprograming in co-inoculated roots. Next, pair-wise comparisons (FDR < 0.01) identified 13,300 genes that were significantly and differentially expressed genes. I separated these genes into 12 different clusters (K-means). Genes grouped into the Clusters 5, 6, 11, 12 were responsive to fungi. GO enrichment analysis indicated that genes related to defense responses such as "response to chitin", "innate immune response" were predominantly enriched in clusters 5 and 12. In clusters 6 and 11, in addition to genes related to defense responses, genes related to Trp metabolism (FDR: 0.004) and ET signaling (FDR: 0.005) were enriched. CgP inoculation induced stronger induction of genes in these clusters compared to CgE inoculation. These results indicate that CgP infection induces stronger transcriptome reprograming that is largely suppressed in the presence of CgE. At the same time, minor changes in plant transcriptome in co-inoculated samples compared with CgE inoculated-samples implies that CgE-mediated plant protection is not dependent on extensive reprogramming of plant transcriptome.

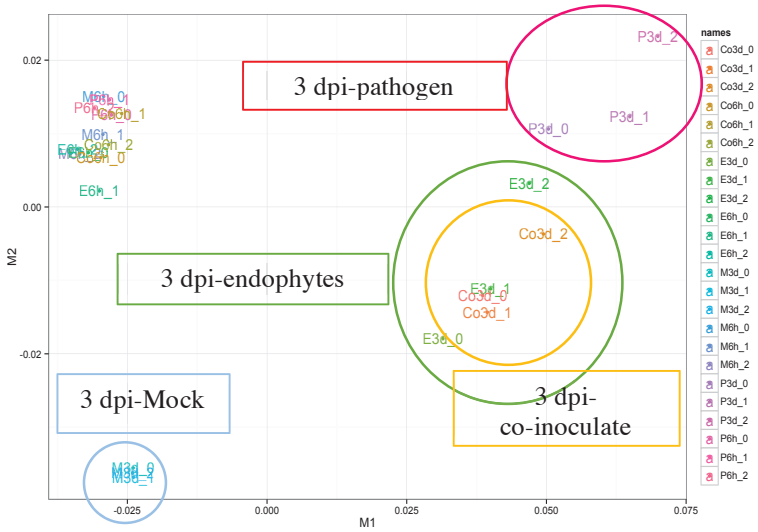


Figure 21. Plant-side transcriptome analysis during root colonization of CgE and CgP. Multi-dimensional analysis chart generated from plant transcriptome profiles during the interactions with CgE and/or CgP at 6 hpi and 3 dpi. CgE and/or CgP at 6 hpi and 3 dpi. M = Mock, E= CgE, P= CgP, and Co = CgE + CgP.

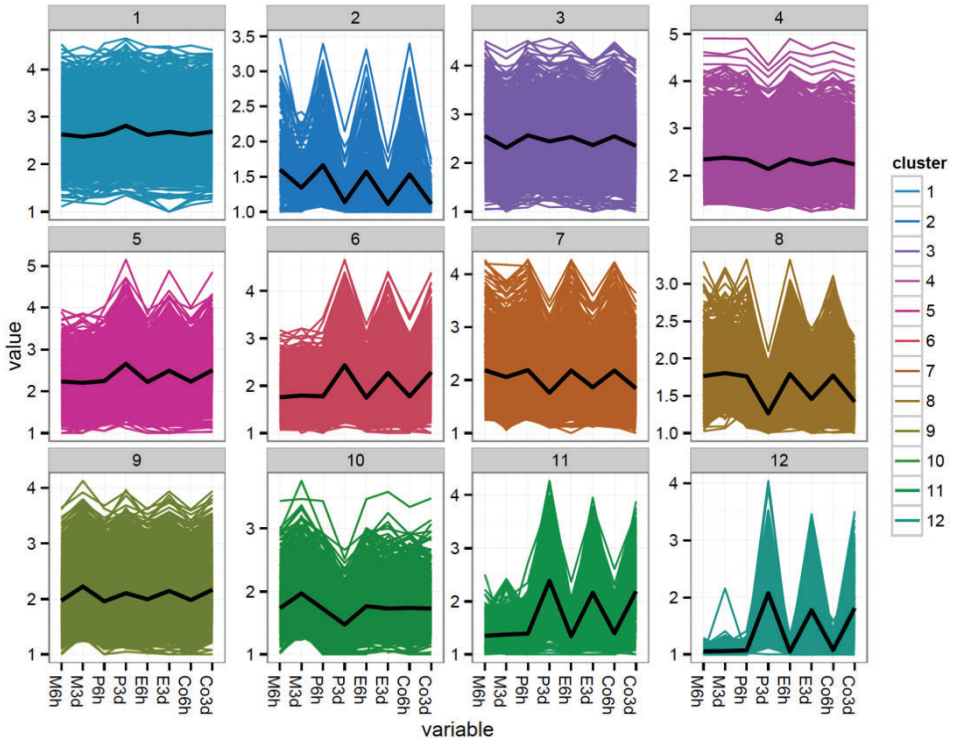


Figure 22. All plant genes significantly and differentially expressed either in one of the pair-wise comparisons (FDR < 0.01). Twelve clusters have been classified by the k-mean method.

Fungal whole genome and transcriptome analyses for CgE and CgP fungi

As mentioned above, I did not detect drastic changes in plant transcriptome upon co-inoculation with CgE and CgP, suggesting that CgE-mediated plant protection does not depend on large-scale transcriptome reprograming of the host plant. One possibility is that CgE-mediated protection depends on gene expression changes on the fungal side. To test this, I together with collaborators first tried to get the whole genome information of CgE and CgP. PacBio long reads and Illumina short reads were used to build high-quality genome assemblies of CgE and CgP. ITS sequences suggested that both fungi are *C. gloeosporioides* (Table 5). However, whole-genome alignment indicates that CgE and CgP are different fungal species, while they are still related taxa within the *Colletotrichum gloeosporioides* species complex. (Figure 23). The predicted genome size of CgP was around 57 Mb, which is comparable to that of the previously sequenced *C. fructicola* (previously described as *C. gloeosporioides*) Nara gc-5 strains (55.6 Mb, Gan et al., 2013). In contrast, the genome size of CgE was 64.5 Mb, and different from that of the other Cg strains (53.2-57.7 Mb). In summary, these results suggest that CgE and CgP are not as closely-related as would have been expected from ITS sequences.

Table 6. Statistic of *Colletotrichum gloeosporioides* CgE and CgP genome assemblies obtained in this study compared to *C. fructicola Nara gc-5* and *C. gloeosporioides gc-14*.

	CgE	CgP	C.f Nara gc-5	C. g gc-14
Assembly size (Mb)	64.5	57.7	55.6	53.2
Number of scaffolds	35	21	1241	4537
Median length (N50)	4923.73 kb	4922.22 kb	112.81 kb	25.33 kb
Gaps	0	0	4094	4537
Number of coding genes	16012	15084	15381	16538

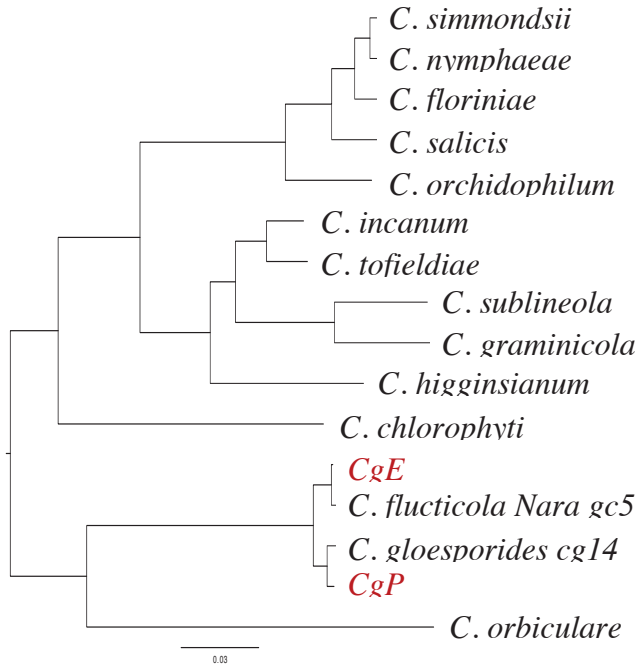


Figure 23. Phylogenetic tree constructed from genome data from 17 *Colletotrichum* spp. using Single copy ortholog (4650 pair)

Dynamic fungal transcriptome changes during fungus-fungus competition in roots

As no drastic transcriptome reprograming was observed between plants inoculated with CgE alone and those co-inoculated with CgE and CgP, I reasoned that changes in fungal transcriptome may explain CgE-mediated host protection. To test this possibility, I tried to profile fungal transcriptome by separating CgE- and CgP-derived sequence reads from the co-inoculated root samples. This analysis revealed that more than a half of the total reads were derived from CgE, while only a few percent of reads were derived from CgP. These results are consistent with predominant colonization of CgE over CgP as shown by fungal biomass measurement (Figure 7). Next, I focused on differentially expressed genes (|log2 FC| >1, FDR < 0.05) between single-inoculated and co-inoculated samples. I detected 892 differentially expressed CgE genes, and 1,239 CgP genes (Table 7). Among the 721 up-regulated CgE genes in co-inoculated samples compared to CgE-inoculated samples were several small secreted proteins (SSPs) genes including those encoding *Colletotrichum*-specific candidate secreted effector proteins (CSEPs) with an N-terminal secretion signal. In pathogenic fungi, a few effector proteins have been reported to suppress plant immunity (Saitoh et al 2012; Irieda et al., 2019). Genes encoding cell wall binding protein, fungal toxin, sugar transporter and antibiotic resistance were also included as up-regulated CgE genes in co-infected samples.

Table 7. Fungal whole genome and transcriptome analyses for CgE and CgP fungi. Number of CgE or CgP genes with differentially expressed in co-inoculated samples compared the single inoculated samples at 3 dpi.

	Up-regulated DEGs	Down-regulated DEGs	Total
Specific CgE genes	721	171	892
Specific CgP genes	564	675	1239

Interestingly, CgE genes related to the biosynthesis and efflux of several different fungal toxins were specifically up-regulated in co-inoculated samples (Table 8). For example, while a fungal toxin gene, *CgBOT2*, was expressed in both CgE and CgP single inoculation, it was highly expressed in CgE but was repressed in CgP after their co-inoculation. I conducted qRT-PCR analysis to verify this conclusion, with CgE-specific primers for *CgEBOT2*. At 3 dpi, expression of *CgBOT2* in the co-inoculated samples was higher than that in the CgE-single inoculated samples (Figure 24), raising the possibility that CgE utilizes a fungal toxin specifically in the presence of CgP *in planta*. This implies a role for the fungal toxin in competition for the limited niches in the host. Overall, the drastic fungal transcriptome changes in co-inoculated roots points to the occurrence and significance of fungus-fungus competition, which seem to enable the protection of host plants without dramatic activation of host defense responses.

Table 8. Up-regulated differential gene expression genes in CgE which related to fungal toxin

Gene	*log Fold Change*
Efflux	
Aspyridones efflux protein	8.12
MFS efflux pump	3.6
Efflux pump	2.9
ABC transporter	2.77
Biosynthesis	
Regulatory protein alcR	4.16
Presilphiperfolan-8-beta-ol synthase (BcBOT2)	4.28
Cytochrome P450 monooxygenase	4.13
Trichothecene C-15 hydroxylase (BcBOT4)	4.05
Transcription Factor	
Secondary metabolism regulator (LAE1)	3.38

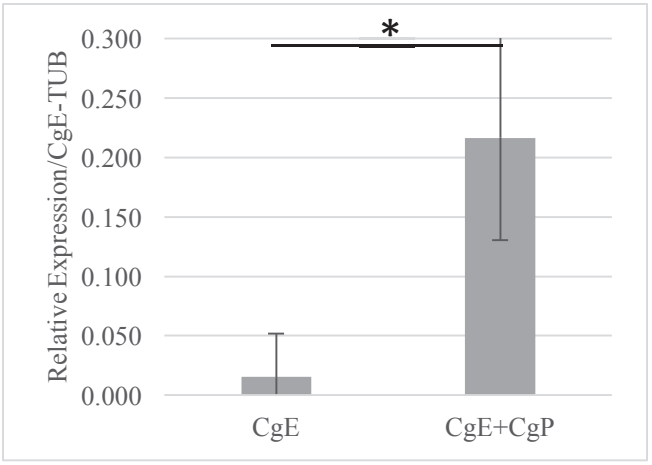


Figure 24. Expression analysis of *CgEBOT2* qRT-PCR at 3 dpi. Each sample consisted of 20-25 roots per experiment. Data were obtained in three independent biological replicates. Bars represent means and standard errors of data collected in all independent experiments. Asterisks indicate significant different means between CgP- and Co-inoculated plants. (T-test, $p<0.05$)

Phosphate availability greatly affects the endophytic lifestyle and plant protection ability of CgE

It has been reported that the root endophyte *C. tofieldiae* promotes plant growth in low Pi conditions but not in normal Pi conditions (Hiruma et al., 2016). To address whether host phosphate availability affects the mutual interaction and plant protection ability of the newly identified *Colletotrichum* endophyte (CgE), I performed a co-inoculation experiment using 1/2 x MS based media containing either sufficient (625 µM) or limited Pi (50 µM). CgP caused severe disease symptoms equally on both Pi sufficient and Pi limited medium. Surprisingly, under low-Pi, CgE caused severe disease symptom on WT plants, as shown with CgP (Figure 25). In addition, the protection ability of CgE was lost under low Pi conditions, implying that CgE becomes pathogenic when phosphate supply is limited. These results suggest that environmental phosphate levels affect the beneficial interaction and the plant protection ability of CgE.

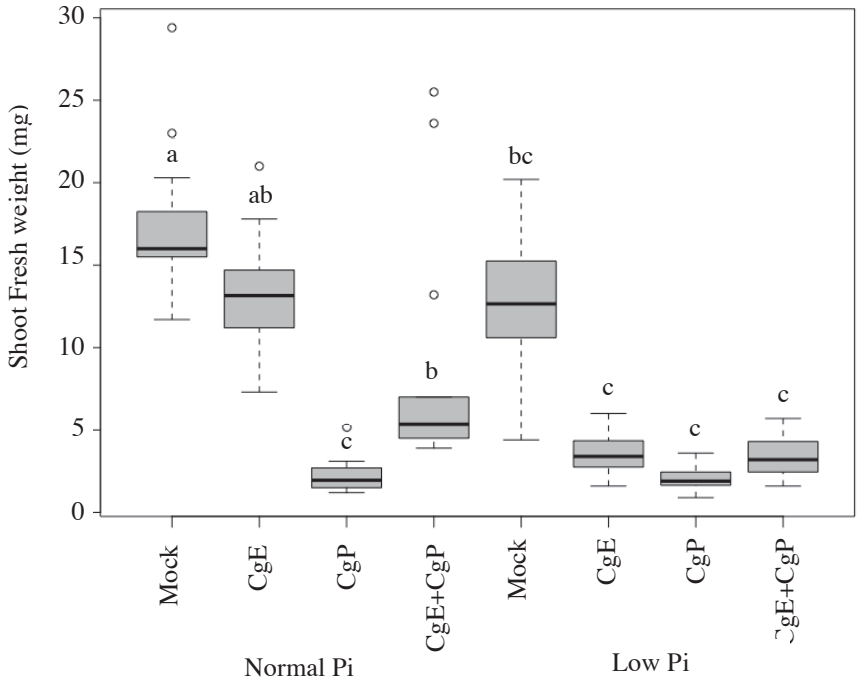


Figure 25. Phosphate limitation affects endophytic lifestyle and plant protection ability of endophytic *Colletotrichum gloeosporioides* (CgE) Shoot fresh weight of *Arabidopsis thaliana (*Col-0) grown in 1/2 x MS media under normal Pi (625 µM) and low Pi (50 µM) conditions 14 dpi with the indicated fungi**.** The boxplot shows combined data from three independent experiments. Different letters indicate significantly different statistical groups. (Tukey-HSD, $p < 0.05$).

DISCUSSION

In this study, I have evaluated the effects of endophytic fungi isolated from *Brassica* plants on the growth of *Arabidopsis thaliana*. My findings show that fungi isolated from healthy *Brassica* plants grown in the same location associate with several beneficial fungi with plant growth promotion function. A number of endophytes in *Brassica* plants have been reported as microbes that provide benefits for their host, such as growth promotion under low-nutrient conditions (Yadav et al., 2010; Hiruma et al., 2016) or induction of host-mediated resistance to pathogens (Kavroulakis et al., 2007; Chalfoun et al., 2011). It is well known that Brassica plants do not associate with arbuscular mycorrhizal fungi, which are considered to be important partners of most of the land plants in plant adaptation to various stress conditions (DeMars & Boerner, 1996). Instead of the interactions, my study again confirms that Brassica plants have developed beneficial associations with non-mycorrhizal fungal endophytes. Interestingly, together with beneficial fungal endophytes, several fungi with severe plant growth inhibition function were isolated from healthy *Brassica* plants. These results suggest that healthy plants in nature can suppress potential pathogenesis of these fungi without apparent activation of plant immune responses. In this thesis, I discuss potential mechanisms underlying the phenomena.

Two *Colletotrichum* fungi from the same clade showed contrasting life-styles in roots

Colletotrichum spp. is a large group of fungus spreading worldwide (Cannon *et al.,* 2012). *Colletotrichum* fungi in Arabidopsis have been reported as both endophytes, such as *C. tofieldiae,* (Hiruma et al., 2016) and pathogens, such as *C. higginsianum and C. incanum* (O'Connell et al., 2012; Hacquard et al., 2016). Two fungi from my isolation have been identified as fungi belonging to *C. gloeosporioides* clade (Table 5), which are well known as pathogens causing anthracnose diseases (Weir et al., 2012). Whole some genomes of the fungi in the *C. gloeosporioides* clade have been previously sequenced and analyzed (Gan et al., 2012). Several studies summarized fungal responses when pathogenic *C. fructicola* (syn. *C. gloeosporioides*) infects in leaves by comparative genomics and transcriptome analyses among several pathogenic *Colletotrichum* fungi during the host infection (Gan et al., 2012; Zhang et al., 2018). In contrast, surprisingly, my plant-fungus co-cultivation experiments indicate that the isolate CgE (E41) was a growth promoting endophyte, while the isolate CgP (E40) was clearly a disease-inducing pathogen, despite that these two fungi were isolated from the same organ (leaf) of healthy radish plant individuals (Figure 1). These results suggest that *Colletotrichum* fungi in the *C. gloeosporioides* clade are diverged in their life-styles from pathogenic to beneficial, which are similar to that in the *Colletotrichum spaethianum* clade of the opposing life-styles from pathogenic (*C. incanum*) to beneficial (*C. tofieldaie*) (Hiruma et al., 2016; Hacquard et al., 2016). Interestingly, CgE can complete its lifecycle in host roots under our conditions as we have observed new generation of conidia and acervulus, which were also detected in CgP colonizing roots. These observations suggest that CgE share some traits with pathogenic CgP in roots. It should be noted, however, that microorganisms in the same clade often display different lifestyles. For example, Melnyk et al (2019) found that closely related bacterial strains of *Pseudomonas fluorescens* within a single clade consisted of both commensal and pathogenic ones.

Beneficial interaction of endophytic *Colletotrichum* fungus relies on host ethylene and tryptophan-derived secondary metabolites

Despite the beneficial function in wild-type plants, CgE turned to be a pathogen in the quadruple hormone-defective (*dde2/ein2/pad4/sid2*) plants, which are simultaneously impaired in responses to JA, ET and SA. This suggests that these components and its downstream components are required for the proper endophytic life-styles of CgE.

Our results show that ET regulates a lifestyle switch of the endophytic *Colletotrichum gloeosporioides* (CgE), from the endophytic to pathogenic mode. ET production can be strongly induced during plant-microbe interactions, e.g. when exposed to fungal toxins or elicitors. ET can activate plant defense responses such as production of pathogenesis-related (PR) protein (Kan et al., 1995), phytoalexin (Fan et al., 2000) and cell wall alterations (Bell, 1981). In Arabidopsis, ET signaling and perception are required in pathogen resistance, especially against necrotrophs (Thomma et al., 1999). On the other hand, ET has been proposed to play an important role in the modulation of host immune responses by beneficial microbes (Zamioudis and Pieterse, 2012). For example, a biological control fungus, *Fusarium solani*, induced plant defense responses through the ET-signaling pathway in order to compete with pathogenic *Fusarium oxysporum* (Kavroulakis et al., 2007). ET also acts in constraining fungal growth by physical reinforcement of root cell walls, possibly to maintain a balance between cost and benefits in the mutualistic plant-fungus interaction (Plett et al., 2014).

ET also organizes the dynamic of cell wall properties in the defense against *Botrytis cinerea* (Ferrari et al., 2007). Downstream and related pathways of ET have been analyzed for identification of critical controller of the plant protection ability of CgE in ET signaling pathway. For example, loss of EIN3 also lost the endophytic interaction with CgE. The less severe disease development compared with *ein2* mutant plants can be attributed to that EIN3 functions together with its homolog EIN3-LIKE 1 (EIL1). Thus, these results indicate the importance of ET signaling in the host plant to prevent *Colletotrichum gloeosporioides* (CgE) from expressing its potential pathogenesis, in part by restricting the fungal growth. This is in sharp contrast to the case of *C. tofieldiae*, where *C. tofieldiae* mediated plant growth promotion is largely unaffected in *ein2 pad4 sid2 dde2* quadruple mutant plants (Hiruma et al., 2016). This suggests that contribution of hormone pathways during plant-endophyte interactions differ even among fungi belonging to the same genus.

A subset of Tryptophan (Trp)-derived secondary metabolites is specially produced by Cruciferous plants, including *Arabidopsis thaliana*, and plays an important role in plant immunity (Tsuji et al., 1992; Bednarek et al., 2009; Bednarek, 2012; Hiruma et al., 2013). Interestingly, otherwise beneficial *C. tofieldiae* causes disease symptoms and eventually kills the *cyp79B2 cyp79B3* plants that lack the cytochrome P450-mediated conversion of tryptophan to indole-3-acetaldoxime (IAOx). In my experiments, CgE also killed the *cyp79B2 cyp79B3* plants. As IAOx is the precursor for the biosynthesis of various active compounds, including the indole glucosinolate and phytoalexin camalexin, which play a role in defense responses (Halkier & Gershenzon, 2006, Sugawara et al., 2009), these results suggest the involvement of these metabolites during the beneficial interaction. Furthermore, *pen2* and *pad3* mutant plants are also hyper susceptible to both CgP and CgE. PEN2 and PAD3 are responsible for the production of indole glucosinolates and phytoalexin camalexin, respectively (Zhou et al., 1999; Ferrari et al., 2003). Some of the compounds have inhibitory effects on the growth of pathogenic fungi *in vitro* (Buxdorf et al., 2013). For example, *Alternaria brassicicola* mycelia treated with camalexin was lysed, therefore leading to the stunned growth (Soledade et al., 2014). Importantly, growth of both CgE and CgP was significantly inhibited when they are directly exposed to camalexin *in vitro*. Therefore, the antifungal pathway is also required for the beneficial interaction between Arabidopsis and CgE, probably via restriction of endophyte overgrowth in roots.

Plant protection by CgE may be a consequence of direct inhibition of CgP by CgE in roots

Some reports have reported that endophytic fungi are able to suppress disease symptoms by pathogens directly and indirectly (Zhang et al., 2014; Dupont et al., 2015). My data shows that CgE has provided a plant protection ability to its host by restricting root colonization of CgP and that of *C. incanum* at lesser extent (Cannon et al., 2012; Hacquard et al., 2016).

Microscopic observation also confirmed that CgP growth was already restricted at an early root colonization phase when co-inoculated with CgE. However, there was no clear direct antagonistic inhibition between these two isolates in dual culture assay, suggesting that CgE requires some host components in order to inhibit CgP. Thus, these results imply that plant tissues and/or signals are required to activate the fungus-fungus competition.

Some of the reported fungal endophytes induce host immune responses to protect plants from pathogens. For example, Kavroulakis et al (2007) have showed that an endophytic *Fusarium solani* is able to protect plants against the root pathogen *Fusarium oxysporum* by the faster root colonization than *F. oxysporum*, and also elicit induced systemic resistance against the tomato pathogen *Septoria lycopersici*. In my study, host transcriptome analysis revealed that CgE and CgP induced similar sets of Arabidopsis genes but at different magnitudes. Genes strongly induced by CgP were partially suppressed when co-inoculated with CgE, which is well correlated with recovered plant growth in the presence of CgE. As expected, some of the significantly up-regulated genes upon CgP and/or CgE challenge were related to plant defense responses, hormone signaling and secondary metabolites pathways. These results suggest that both endophyte and pathogen induce defense-related genes but at different level, which are consistent with the reported work showing that pathogenic *C. incanum* induced stronger defense responses than beneficial *C. tofieldiae* did (Hacquard et al., 2016). However, at the same time, our plant-side transcriptome analysis has revealed that CgE provides host protection from CgP apparently without further activation of plant defense-related gene expression responses. These results suggest that CgE-mediated plant protection is not mediated by further host transcriptome reprograming in the presence of the pathogen. As similar, it has been reported that the root endophyte *S. vermifera* protects barley from *B. sorokiniana* without any detectable host large transcriptome reprograming (Sarkar et al., 2019). The authors have proposed that the endophytic *S. vermifera* infects its prey as a mycoparasite largely irrespective of the presence of host plants. In contrast, CgP growth inhibition by CgE seems to predominantly occur when both fungi are in the same hosts. Importantly, in contrast to relatively stable plant transcriptome in co-inoculated samples, the fungal transcriptomic analyses based on their newly obtained whole genome information have revealed that more than 500 CgE genes are highly activated only in co-inoculated samples. This is also sharp contrast to the root endophyte *S. vermifera* whose fungal transcritpome does not also significantly change by the presence of pathogenic *B. sorokiniana* (Sarkar et al., 2019). Interestingly, the list includes potential fungal genes related to biosynthesis or secretion of several different fungal toxins. Fungal toxin or mycotoxin are a group of fungal secondary metabolites which are produced in a specific condition, such as during host infection or defense against other microbes. From the fungal global transcriptomic analysis, the CgE fungal toxin genes with a homology to *B. cinerea BcBOT2* was highly expressed when co-inoculated with CgP. *BcBOT2* was originally found in the plant necrotrophic pathogen *Botrytis cinerea,* and were described as one of the virulence genes responsible for fungal toxin conversion during plant infection (Pinedo et al., 2009). In my study, I found that CgE specifically induced the gene in co-inoculated roots, without causing plant growth retardation. Thus, these results suggest that CgE may utilize the product from its *CgEBOT2* as a weapon not to fight with host plants but to fight with CgP, a relative pathogen in roots, which eventually lead to the host protection. If this is the case, this idea provides us with a new role of fungal toxins for fungus-fungus competition in host roots. It would be very important to test whether knockout of the gene could influence CgE-mediated CgP suppression in roots. Recently, it has been reported that commensal bacteria in mucosa inhibit and kill the pathogenic relatives in the same genus by releasing DNA into the environment (Kim et al., 2019). For pathogen killing, it is necessary to let the DNA with a specific methylation pattern uptake by the pathogen. The emerging several unique strategies of beneficial and/or commensal microbes to eliminate the pathogen

relatives may represent their fierce fights over essential components (e.g., nutrients) whose requirements are often shared with pathogens due to their close relationship.

Alternatively, but not mutually exclusively, since CgE is more tolerant to anti-fungal phytoalexin Camalexin compared with CgP *in vitro* as described above, CgE-mediated protection might be linked with the fact that CgE is more adapted to Arabidopsis roots in which production of camalexin and possibly other antifungal metabolites is highly activated. Recently, it has been reported that root-specific camalexin biosynthesis mediated by a cytochrome P450 CYP71A12 controls the beneficial functions of several root-associated bacteria (Koprivovoa et al., 2019). Since the activity of CYP71A12 varies among *A. thaliana* accessions, plants may use the anti-microbial metabolite to modulate their own root-associated microbiome, depending on changing environments.

Phosphate low availability turns CgE to a pathogen

Phosphate is one of the essential macronutrients which limit plant growth. The availability of phosphate in the substrate also affects the lifestyle of endophytes. Some endophytes such as *Piriformospora indica and* a Helotiales fungus, which are able to promotes plant growth of Brassicaceae plants under both phosphate limiting and sufficient conditions (Yadav et al., 2010; Almario et al., 2017). In contrast, *C. tofieldiae* which live as an endophyte with an ability of phosphate transfer to its host only under phosphate limiting conditions (Hiruma et al., 2016). In phosphate limiting conditions, unlike *C. tofieldiae*, CgE turned to a pathogen in low-Pi conditions. Moreover, CgE as mention earlier shows plant protection in phosphate sufficient conditions, but losses this ability in phosphate limiting conditions. In contrast, *C. tofieldiae* lacks such plant protection ability against CgP irrespective of phosphate nutrient conditions. Thus, these results suggest that phosphate availability largely but differentially influence the beneficial function of the endophytic *Colletotrichum* fungi in Arabidopsis host.

Conclusion

Currently, several fungi with host protection function have been reported. However, the molecular mechanisms underlying the protection function are still largely unclear. I have demonstrated the newly identified endophytic *Colletotrichum* spp. and its protection ability against pathogenic fungi, without causing plant growth retardation. My study has identified two different host genetic modules that are required for the beneficial interactions between the endophyte and host plants. Based on fungal transcriptome analysis during protection, it further indicates that CgE-mediated host protection is rather a consequence of its direct competition with a pathogen, possibly with toxin-based fungal growth inhibition that is induced in plants Furthermore, unlike the recently reported beneficial fungal endophyte *C. tofieldiae*, CgE has unique features that their potential virulence is regulated by a plant phosphate availability. The *Colletotrichum - A. thaliana* interaction offers a new model for diverse beneficial endophytic fungus-plant interactions that remain poorly understood, thanks to the amenability of the fungal partners to molecular genetic manipulation and the wealth of genetic tools and resources available for the host plant.

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