A novel Arabidopsis pathosystem reveals cooperation of multiple hormonal response-pathways in host resistance against the global crop destroyer *Macrophomina phaseolina*

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Dubbed as a “global destroyer of crops”, the soil-borne fungus *Macrophomina phaseolina* (*Mp*) infects more than 500 plant species including many economically important cash crops. Host defenses against infection by this pathogen are poorly understood. We established interactions between *Mp* and *Arabidopsis thaliana* (Arabidopsis) as a model system to quantitatively assess host factors affecting the outcome of *Mp* infections. Using agar plate-based infection assays with different Arabidopsis genotypes, we found signaling mechanisms dependent on the plant hormones ethylene, jasmonic acid and salicylic acid to control host defense against this pathogen. By profiling host transcripts in *Mp*-infected roots of the wild-type Arabidopsis accession Col-0 and ein2/jar1, an ethylene/jasmonic acid-signaling deficient mutant that exhibits enhanced susceptibility to this pathogen, we identified hundreds of genes potentially contributing to a diverse array of defense responses, which seem coordinated by complex interplay between multiple hormonal response-pathways. Our results establish *Mp*/Arabidopsis interactions as a useful model pathosystem, allowing for application of the vast genomics-related resources of this versatile model plant to the systematic investigation of previously understudied host defenses against a major crop plant pathogen.

The broad host-spectrum pathogen *Macrophomina phaseolina* (*Mp*) is a devastating soil-borne fungus that infects more than 500 plant species1–5. Many of these hosts are economically important crop plants including maize, soybean, canola, cotton, peanut, sunflower and sugar cane5–10. *Mp* is found throughout the world11–15, notably in warmer regions where crop diseases caused by the pathogen are typically associated with drought and heat stress16. Increasing numbers of first reports of *Mp*-caused crop diseases6,9,12 combined with the possibility of global warming effects benefitting the spread of this pathogen through stressed hosts makes the need to effectively combat this pathogen imperative.

*Mp* forms mycelia with microsclerotia imbedded in the hyphae17. A microsclerotium is an aggregation of 50–200 hyphal cells that form a compact mass and is generally brown to black in color when fully formed. Microsclerotia are asexual (non-sporic) propagation structures that may remain dormant for extended periods of time (depending on environmental conditions) prior to germination. Hyphae extend from microsclerotia, typically form appressoria upon contact with host plant tissues and penetrate plant dermal cells growing intra- and

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inter-cellularly in roots and other tissues\textsuperscript{18,19}. \textit{Mp} hyphae produce cell wall degrading enzymes (CWDEs) and phytoalexins\textsuperscript{20} and typically colonize vascular tissue resulting in plant wilting and often death\textsuperscript{7,21}. Although mainly described as a necrotroph, \textit{Mp} appears, in some cases, to exhibit a biotrophic phase early in its infection cycle\textsuperscript{22}, and may be more correctly referred to as a hemibiotroph.

\textit{Mp} microsclerotia can persist in soil for weeks to several years and are extremely difficult to eradicate\textsuperscript{23,24}. Methods of \textit{Mp} management include soil solarization, soil fumigation, no-tilling, crop rotation, flooding, fungicide and other soil amendments (including bio-agents and chemical additives) and seed treatments but the reduction in pathogen development is often only partial\textsuperscript{25–31}. In addition, fungicidal treatments pose environmental health risks\textsuperscript{8}. Field observations have suggested that \textit{Mp} infections are rising because of restriction of the use of methyl bromide\textsuperscript{27,32,33} and alternative methods for \textit{Mp} eradication are urgently sought.

Recent research with crop plants has contributed valuable information using \textit{Mp}-susceptible-versus-resistant variety phenotyping\textsuperscript{19,34} and genetic studies\textsuperscript{35–37}. This ongoing work is conducted in the field, greenhouse and laboratory. Semi-\textit{in vitro} assays, such as those conducted by Bressano and coworkers\textsuperscript{38} and Chowdhury and coworkers\textsuperscript{39}, have been utilized to observe the \textit{Mp} infection process in the roots of certain crop plants. The Bressano group examined early infection of \textit{Mp} hyphae in soybean roots\textsuperscript{40} while Chowdhury and coworkers observed \textit{Mp} behavior in the vicinity, on the surface and inside sesame tissues and were able to quantify microsclerotia in sesame roots\textsuperscript{39}.

Major strides to identify the specific genetic regulatory mechanisms involved in conferring resistance are still needed and the design of innovative strategies for efficient protection of crops against \textit{Mp}-caused diseases requires detailed and comprehensive knowledge of host immune responses directed against this pathogen. The model plant \textit{Arabidopsis thaliana} (Arabidopsis) has been a key research tool, both to gain information about plant defense and to analyze the infection process in a compatible pathogen\textsuperscript{39,40}. It is unparalleled in its stable genetic transformation capability among multicellular organisms\textsuperscript{41}. Its genome was fully sequenced almost two decades ago, large sets of Arabidopsis mutants with well-characterized defects in processes mediating immunity are available and valuable genomics-related resources have been developed and extensively used by the scientific community for over 20 years. As a result, an extensive body of knowledge on the molecular genetics, biochemistry and physiology of the immune system of this organism has accumulated. Various types of immune receptors, signaling components and transcription factors have been identified as important for pathogen defense in Arabidopsis\textsuperscript{42–44}. Arabidopsis immune responses have further been found to be controlled by the defense hormones salicylic acid (SA), ethylene (ET) and jasmonic acid (JA). While SA is known to mainly mediate host immunity against pathogens with biotrophic lifestyles, ET and JA mediate protection against necrotrophic pathogens and herbivorous insects\textsuperscript{45,46}. Surprisingly, to our knowledge, quantitative studies on interactions of Arabidopsis with \textit{Mp} have not been reported in research articles and this versatile plant model system has not been deeply exploited for studies on host defense against \textit{Mp}.

Here we report on a semi-\textit{in vitro} assay system to study Arabidopsis/\textit{Mp} interactions. Our assays allow for accurate quantitative assessment of \textit{Mp} biomass growth in Arabidopsis roots and the extent of host disease symptoms in aerial plant parts. We found Arabidopsis mutants compromised in ET, JA and/or SA signaling to exhibit enhanced susceptibility to \textit{Mp}. This effect was particularly robust in the ET/JA deficient \textit{ein2/jar1} double-mutant. Profiling \textit{Mp}-induced transcriptional responses in this line and its parental wild type background, Col-0, by RNA-seq, linked transcriptional up-regulation of multiple known JA, ET and SA response-regulators to immunity against this pathogen. Results described here will serve as a basis for more extensive systematic studies on \textit{Mp} defense responses in the Arabidopsis model system and provide candidate genes to be further tested for their contribution to \textit{Mp} protection in Arabidopsis and in agricultural crop plants.

**Results**

**A quantitative assay system for \textit{Mp}-infected Arabidopsis roots.** A fundamental step in the infection of plants by \textit{Mp} is the establishment of hyphae and microsclerotia in plant tissue. We observed this process in \textit{Mp}/Arabidopsis interactions on agar plates. \textit{Mp} microsclerotia added to \(\frac{1}{2}\) MS agar generate hyphae that grow throughout the agar, forming more microsclerotia as \textit{Mp} grows. When Arabidopsis seedlings are laid down upon the \textit{Mp}-laden agar infection plate, hyphae on the agar surface contact the roots and grow toward them, surrounding and penetrating the root tissue (Fig. 1A,D). Microsclerotia can be seen forming within the roots as early as 48 hours post contact (hpc) in Arabidopsis (Fig. 1E).

The fact that critical events of the \textit{Mp} infection process occur in Arabidopsis roots under controlled conditions on agar enables the design of plate-based infection protocols to accurately quantify levels of host susceptibility (Supplementary Fig. S1). Here, Arabidopsis seeds were sown on \(\frac{1}{2}\) MS agar plates (Supplementary Fig. S1A) and allowed to grow for ten days (Supplementary Fig. S1C) while \textit{Mp} microsclerotia were added to separate plates (Supplementary Fig. S1B) and grown for six days (Supplementary Fig. S1D). The ten-day-old seedlings were transferred to the six-day-old \textit{Mp} infection plates (Supplementary Fig. S1E). After 24 hours of contact (Supplementary Fig. S1F), the seedling roots were infected by \textit{Mp} hyphae (Fig. 1A,C). By 48 hours, hyphae penetration continued and microsclerotia could be seen forming in some Arabidopsis roots (Fig. 1E). Root tissue was flash frozen in liquid nitrogen at 24 hpc and 48 hpc for RNA-seq or at 48 hpc for qPCR analysis (Supplementary Fig. S1H). Alternatively, at four-five days post contact (dpc) microsclerotia density was determined by counting the number of microsclerotia per mm in each seedling’s primary root (Supplementary Fig. S1G). Infection plates typically contained 12–14 seedlings, and the microsclerotia per mm density was based on the average of all tested seedlings per plant line (at least three plates per line) in a biological replicate.

**Arabidopsis mutants compromised in ET signaling show enhanced root susceptibility to \textit{Mp}.** To identify host defense mechanisms that affect levels of \textit{Mp} susceptibility in Arabidopsis roots, we tested a set of Arabidopsis Col-0 mutants. As \textit{Mp} is known to mainly exhibit a necrotrophic lifestyle, we tested the \textit{jar1} and
ein2 single mutants as well as the ein2/jar1 double mutant with defects in signaling processes mediated by JA or/and ET, respectively (Fig. 2A). Mutants of JAR1 (Jasmonoyl isoleucine conjugate synthase1) are compromised in the conversion of \(+\)-7-iso-JA to \(+\)-7-iso-jasmonoyl-L-isoleucine (JA-Ile), which is one of the main bioactive forms of JA.\(^47\) The jar1 mutant is known to exhibit complete loss of JA signaling in Arabidopsis roots.\(^48\) The ER-associated EIN2 (ET INsensitive 2) protein links signaling processes triggered by various ET receptors to EIN3-EIL1-type transcription factors.\(^49\) Null mutants of EIN2, including the ein2 lines used here, are completely ET insensitive.\(^50\)

In the ein2/jar1 double mutant, but not in the ein2 or jar1 single mutants, we observed significantly increased density of microsclerotia in roots (Fig. 2A). We confirmed this finding by comparing the abundance of Arabidopsis- and Mp-specific genomic DNA in Arabidopsis roots 48 h after Mp contact using quantitative PCR (qPCR) with primer pairs specifically targeting species-specific sequence characterized amplified regions (SCAR) of Mp (MpSyk) and Arabidopsis (Shaggy-related Kinase 11, AtSK11) DNA.\(^51,52\) (Fig. 2B). This SCAR-qPCR assay showed relative abundance of Mp-specific DNA (compared to Arabidopsis-specific DNA) to be clearly elevated in Mp-infected roots of the ein2/jar1 line compared to Col-0 plants indicating twice the amount of Mp biomass in this mutant. Contrary to our microsclerotia density assay, we also observed a significant increase of relative Mp DNA levels in the ein2 single mutant compared to Col-0 (Fig. 2B). We attribute this to the fact that the SCAR-qPCR assay is more sensitive than microsclerotia density measurements and detect DNA from other Mp structures, such as hyphae. While neither the microsclerotia density assay (Fig. 2A), nor SCAR-qPCR (Fig. 2B) indicated significantly enhanced susceptibility in the jar1 single mutant, this mutant exhibited a large degree of

**Figure 1.** Mp infected Arabidopsis root tissue at 24 and 48 hours post-contact (hpc). (A,D) Mp hyphae surrounded and penetrated Arabidopsis roots at 24 (A) and 48 (D) hpc. (B) Mp microsclerotium (black arrow), nearly in the microscopic focal plane with the root, shows relative size reference. (C) Bracket indicates a single root hair surrounded by hyphae at 24 hpc. (E) Microsclerotia were forming inside root tissue at 48 hpc. (F) The black arrowhead points to hyphae emanating from the microsclerotium. The white arrowhead points to mature hyphae. (G,H) Acid fuchsin stained Mp-infected Arabidopsis roots. Mp microsclerotia began to form as early as 48 hpc throughout root tissue and were often associated with vascular tissue. White arrows point to locations of hyphal insertion into root tissue. Black arrows point to microsclerotia. Bracket indicates vascular bundle, vb; vascular bundle, x; xylem cells, lr; lateral root.
Mp biomass variability in the SCAR-qPCR. We also included, in both assays, the transgenic NahG line53 which does not accumulate the defense hormone SA, but did not observe any significant effect. However, as in the case of jar1, our SCAR-qPCR assay revealed that Mp biomass variability was unusually strong in NahG plants (Fig. 2B).

Collectively, in our agar plate-based assay system, we observed enhanced levels of Mp growth in mutants compromised in ET signaling, showing that this stress signaling pathway contributes to defense reactions in Arabidopsis roots against Mp.

Arabidopsis mutants compromised in ET-, JA- and SA signaling show enhanced susceptibility to Mp in shoots. In order to investigate Mp-induced damage to aerial plant tissues, since shoot damage is a common visual indicator of disease in many Mp-infected crop plants in the field54, we repositioned the seedlings and increased the length of time on Mp infection plates. Despite the still-abundant level of Mp on infection plates, these few changes allowed for a more gradual development of disease symptoms with visible phenotype variation in shoots (i.e., differing rates of decay) between lines (Fig. 3). Similar to the root assays, ten day old Arabidopsis seedlings were transferred to Mp infection plates; however, plants were allowed to grow for two weeks with plates positioned right-side-up under growth room conditions. Seedlings were photographed during the infection period and their disease severity was determined. Using a disease index scoring system of “0”, for healthy plants, through “5”, for dead plants, (Fig. 3B), we compared disease symptoms (e.g., chlorosis, necrosis, stunting) between Col-0 and mutant lines. Of the six mutants with defects in ET and/or JA-signaling that we tested, five (ein2/sid2, ein2, ein3/eil1, ein2/jar1, jar1) exhibited more severe disease symptoms (average disease index scores of 4.5, 4.3, 4.0, 4.6, 3.3, respectively) than Col-0 (wild type, average disease index score: 2.26) after two weeks (Fig. 3C). In some cases, the effect was already clear after 1 week. After two weeks of Mp infection, ein2/jar1 had the most severe disease symptoms and many of the ein2/jar1 (81%), ein2 (70%) and jar1 (34%) plants were dying (disease index score 4.5–5; Col-0, 7%) (Fig. 3A). In addition to these mutants, which are compromised in upstream ET- and/or JA-signaling processes, we also observed clearly enhanced Mp susceptibility in the ein3/eil1 double mutant (Fig. 3C), which is deficient in EIN3 and EIL154. Both of these related transcription factors control almost all ET-responses as well as some responses to JA55. However, we did not observe any significant change of Mp susceptibility levels in jin1, a mutant deficient in the transcription factor Myc2, which mediates JA-triggered wound responses56.

Shoot disease symptoms were less severe in SA-deficient NahG plants than in those of the JA and ET mutants, yet were also significantly higher than Col-0 plants at the two-week time point (Fig. 3C). To confirm a potential role of SA in host defenses against Mp we tested additional SA signaling deficient Arabidopsis lines and observed enhanced levels of Mp susceptibility relative to Col-0 in the sid2-2, wrky70 and wrky70/54 mutants (Fig. 3C). The sid2-2 mutant is deficient in isochorismate synthase 1, an enzyme catalyzing a key step in defense-induced SA biosynthesis57, while the tested wrky mutants are blocked in regulatory steps downstream from SA, with WRKY70 and WRKY54 being two closely related TFs mediating SA-responsive gene expression58,59. Given that SA contributes to elevated Mp tolerance in our experiments, the lack of significant effects of npr1 and pad4 plants

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Figure 2. Mp growth quantification using an agar plate pathosystem and SCAR-qPCR identified mutant plant line(s) with increased susceptibility to Mp. (A) Mp microsclerotia were counted under microscopy in individual seedling primary roots, 4–5 days post-contact (dpc). The average percent microsclerotia per mm root relative to Col-0 is shown. (B) The relative abundance of Mp and Arabidopsis genomic DNA was determined by the quantification of sequence characterized amplified regions (SCAR) of Mp (MpSyk)51 and Arabidopsis (Shaggy-related Kinase 11, AtSK11)52 by qPCR using Arabidopsis roots 48 hpc with Mp. Error bars represent standard error for three biological replicates. Statistical significance relative to Col-0 determined by Student’s t-test, asterisk; p < 0.05.
in the shoot disease index assay was unanticipated. Yet, while NPR1 and PAD4 are both known to contribute to SA-dependent immunity, their significance for certain defense mechanisms seems variable. NPR1 is indispensable for SA-dependent systemic immunity, but its importance for local defense reactions may be limited. The
Our comparison shows 54% (449/827) of a set of Arabidopsis genes induced by JA 67 overlap with -induced responses to those activated by other defense stimuli.

A comparison of the same set of Arabidopsis Col-0 genes up-regulated in response to Mp infection at either 24 hpc or 48 hpc (3,396 genes) seems to a certain extent unique, as it shows only partial overlap with gene sets up-regulated by other pathogens, such as the necrotrophic fungi Fusarium oxysporum and Botrytis cinerea or the biotrophic oomycete Hyaloperonospora arabidopsidis (Hpa) (Fig. 4A). Of the three comparisons in Fig. 4A, the gene set induced by F. oxysporum has the largest relative overlap with the Mp-induced transcript profile in Col-0 roots, as 68% (79/116) of F. oxysporum-induced genes are also up-regulated by Mp. F. oxysporum and Mp are both necrotrophic, soil-borne fungal pathogens that often infect plants through their roots. The overlap of genes up-regulated in aerial parts of Arabidopsis by the necrotrophic fungus B. cinerea with Mp-induced genes is less extensive at 42% (687/1,637), which, however, may partially reflect the fact that different types of host tissues were used. A relatively low percentage (28%; 1,123/3,950) of Arabidopsis genes induced by Hpa are also up-regulated by Mp. This is not surprising given that Hpa is a biotrophic oomycete that infects aerial portions of the plants and exhibits a very different lifestyle from Mp. Again, these differences also likely reflect that different host tissue types were profiled. Despite the fact that the relative overlap of these two gene sets is small, a large absolute number of genes (1,123) are jointly up-regulated in both types of infections indicating that some host defense responses may be inducible by both of these dissimilar types of pathogens.

Similarly, a comparison of the same set of Mp-induced genes to those up-regulated in hormone-treated Arabidopsis revealed commonalities of Mp-induced responses to those activated by other defense stimuli (Fig. 4B). Our comparison shows 54% (449/827) of a set of Arabidopsis genes induced by JA overlap with our Mp inducible gene set. Of 177 genes induced in Arabidopsis by the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC), 67, or ~38%, overlap with the set of Mp –up-regulated genes (Fig. 4B), while, of 590 Arabidopsis genes up-regulated by the SA analog INA, 203 (34%) of which overlap with Mp inducible genes. These observations further confirm a likely role of ET and JA as critical regulators of immune responses against Mp, but also suggest that parts of the canonical SA-dependent defense system also may be activated in response to Mp infections.

**Promoter-motif enrichment analysis with Mp-responsive gene sets.** Enrichment of sequence motifs representing certain transcription factor (TF) binding sites in promoter regions of Mp-responsive genes further supports a combined role of JA, ET and SA in controlling transcriptome changes induced by this...
pathogen. TFs typically bind specifically to short DNA stretches of defined sequence, but also exhibit some ambiguity binding to a range of derivatives of a certain type of DNA motif. While any sequence motif is randomly distributed in genomic DNA, statistical enrichment of a given motif in promoter regions of genes that are co-expressed under certain conditions, can indicate a role for this motif (and cognate binding TFs) in directing this expression pattern.°
JA-responses are often mediated by Myc2 and related bHLH TFs, which have a strong binding preference for the well-characterized G box (CAGGTG)\textsuperscript{6,7,1}. Transcriptional up-regulation in response to ET and JA is mediated by the related Arabidopsis TFs EIN3 and EIL1\textsuperscript{4,5}. EIN3 (and likely also EIL1) can bind to DNA sequences containing the pentameric motif TACAT\textsuperscript{7,3}. In response to ET and/or JA, EIN3/EIL1-related TFs can activate expression of members of the large ERF TF family which bind to a variety of binding sites related to the GCC box (AGCCGCC)\textsuperscript{7,3}. WRKY TFs, which bind to various sequences related to the W box (TTGACC/T)\textsuperscript{8,9}, are associated with SA-driven transcriptome changes, but can also be involved in other types of immune responsive gene expression control, such as responses mediated by PTI-associated MAP-kinase cascades. More strictly associated with SA-signaling seem to be TGA-hZIP TFs and TBF1\textsuperscript{7,5,7}. While the former type of TFs are known to bind to TGA boxes (TGACG), TBF1 (and likely additional so far unknown factors) binds to TC-rich sequences, such as the TLI1 element (TTCTTCTCT)\textsuperscript{7,6} or the related LURP\textsuperscript{7} element (TTCTTCTCT)\textsuperscript{8}. Our set of RNA-seq data allowed us to discriminate three temporal patterns of Mp-induced transcript accumulation in Col-0 plants: (1) “early/transiently up” (only up-regulated by Mp at the 24 h time point), (2) “early/sustained up” (up-regulated at both 24 h and 48 h after Mp contact) and (3) “late up” (only up-regulated at 48 h after Mp contact). We observed strong enrichment of hexameric motifs related to all tested promoter motifs in our sets of Mp-response genes, except for the GCC box (Table 2). While in the promoter regions of “early/transiently up”-regulated genes only a G-box related motif seems conserved, the two remaining sets, “early/sustained up” and “late up”, feature a variety of conserved motifs related to the G-box, EIN3/EIL1 binding sites, W-box, TGA box and TC-rich motifs. Thus, the up-regulation Mp-responsive genes may indeed be mediated by the combined activity of known ET/JA-associated TFs (Myc2-like, EIN3/EIL1-like) as well as SA-associated (WRKY, TGA-hZIP and TC-rich motifs) TFs. These observations are consistent with the enhanced Mp susceptibility phenotype we observed in ein3/eil1 and wrky70/74 mutants and further support that host immune responses of Arabidopsis against Mp are mediated by the combined activity of ET/JA signaling and SA signaling pathways.

Despite the fact that G box-related motifs are strongly enriched in the examined sets of Mp-induced Arabidopsis genes, we had not observed altered Mp-susceptibility of the Myc2-deficient jin1 mutant. This may reflect the complex roles of Myc2 in JA/ET-signaling, as it can suppress certain groups of genes, while activate others\textsuperscript{8,9}. Lack of an Mp defense-related phenotype in jin1 may also be due to possible functional redundancy between this TF and other related Myc family members\textsuperscript{7,7}.

**Functional categories of Mp-responsive Arabidopsis genes.** We further categorized all Arabidopsis genes differentially expressed after Mp based on the timing and dependency of their transcriptional changes on EIN2/JAR1 into 12 groups (labeled by roman numerals I - XII in Fig. 4E,F, Supplementary Tables S13–S25) and performed a GO term enrichment analysis. Differential gene expression changes detected by our RNA-seq analysis may reflect inducible host defense responses to combat Mp and active host responses to counter Mp-inflicted disease symptoms and damage. Furthermore, such gene expression changes may also be the consequence of Mp effector activity and serve pathogen virulence rather than host immunity and damage protection. Several general themes stand out. Consistent with our observation that known defense responses mediated by JA, ET and SA contribute to host resistance against Mp, groups of Mp-up-regulated genes are enriched for GO terms associated with responses to defense, stress, JA, ET, SA, various pathogens and, also, wounding (a known trigger of JA signaling). Similarly, up-regulated genes associated with the strongly enriched GO term “camalexin biosynthesis” are likely serving direct host immune responses, by promoting the synthesis of this indole-related phytoalexine. Host genes up-regulated to counter Mp-inflicted damage may be associated with GO terms representing protein re-folding, senescence, and responses to starvation, heat, or toxic substances. Using the set of assays, we also found Mp susceptibility to be enhanced in the ein2 and jar1 single mutants, which are deficient in responses to ET or JA, respectively. These results, and observations we made with additional ET- or JA-mutants, implicate ET and JA signaling as important for protection against Mp in Arabidopsis. Together, the assays can uncover multiple plant responses throughout the Mp infection process. ET-related responses are clearly evident in roots at 24 h (RNA-seq data), at 48 h (RNA-seq data and SCAR-qPCR Fig. 2B) and at 4–5 dpc (as part of the ein2/jar1 microsclerotia density increase) as well as in shoots at 7 and 14 dpc. JA-related responses also contribute to root susceptibility, for while there was moreMp tissue present in the ein2 plants than in the jar1 plants (SCAR-qPCR Fig. 2B), microsclerotia density only increased when both signaling pathways were compromised (see ein2/jar1 Fig. 2A), suggesting a possible relationship between JA signaling and microsclerotia formation that merits future investigation. In the shoot, ein2 was significantly different from ein2/jar1 at 14 dpc, though not at 7 dpc (data not shown), pointing to more of a role for JA during the later infection period.
| Gene set                                            | Conserved motif(s) (Segments matching known motif consensus are underlined.) | Motif type/general motif consensus | p value/comments |
|---------------------------------------------------|-----------------------------------------------------------------------------|-----------------------------------|-----------------|
| early/transiently up (47 genes up-regulated in Col-0 by Mp ONLY at 24 hpc AND NOT at 48 hpc) | ACGTGTCG (G box-like (CACGTG))                                               | p = 4.00e-04 (top hit, 19 matches) |
|                                                   | TTAGGC (W box (TTGACC/T))                                                    | p = 1.10e-07 (top hit, 143 matches) |
| early sustained up (213 genes up-regulated in Col-0 by Mp at both 24 hpc AND 48 hpc)      | ACGTGTCG (G box-like (CACGTG))                                               | p = 9.28e-07 (78 matches)          |
|                                                   | TGACCT (G box-like (TTGACC/T))                                               | p = 1.99e-06 (123 matches)         |
|                                                   | TGACCT (G box-like (TGACG))                                                  | p = 6.99e-06 (93 matches)          |
| only late up (521 genes up-regulated in Col-0 by Mp ONLY at 48 hpc AND NOT at 24 hpc)       | ACGTGTCG (G box-like (CACGTG))                                               | p = 1.02e-12 (top hit, 201 matches) |
|                                                   | CACGTA (G box-like (CACGTG))                                                 | p = 2.91e-07 (142 matches)         |
|                                                   | TAAACAT (EIN3/EIL1-binding site like (ATCAT))                                | p = 5.27e-07 (362 matches)         |
|                                                   | ACGTGTCG (G box-like (CACGTG))                                               | p = 8.19e-07 (162 matches)         |
|                                                   | ACGTGTCG (G box-like (CACGTG))                                               | p = 3.72e-05 (155 matches)         |
|                                                   | CTCTTC (TLI/LURP, TC-rich motifs TCTTCT)                                     | p = 3.89e-05 (239 matches)         |
|                                                   | CTCTCA (TLI/LURP, TC-rich motifs TCTTCT)                                     | p = 2.30e-04 (296 matches)         |
|                                                   | CTCTCG (TLI/LURP, TC-rich motifs TCTTCT)                                     | p = 3.23e-04 (149 matches)         |
|                                                   | AACACAT (EIN3/EIL1-binding site like (ATCAT))                                | p = 6.90e-04 (428 matches)         |
|                                                   | AACACTT (EIN3/EIL1-binding site like (ATCAT))                                | p = 6.98e-04 (402 matches)         |
|                                                   | CTCTTC (TLI/LURP, TC-rich motifs TCTTCT)                                     | p = 7.76e-04 (230 matches)         |
|                                                   | GTGTCG (W box-like (TTGACC/T))                                               | p = 8.43e-04 (193 matches)         |
|                                                   | TGACCG (TGACG)                                                              | p = 9.89e-04 (132 matches)         |
|                                                   | ACGTGTCG (G box-like (CACGTG))                                               | p = 8.57e-06 (103 matches)         |

Table 2. Enrichment of hexameric sequence motifs representing defense-associated transcription factor (TF) binding sites in promoter regions of genes responsive to Mp in Col-0 plants. Known sequence motifs considered in this analysis (listed with their cognate TF family in bold): CACGTG (G-box, *Myc2-like*), ATCAT (*EIN3/EIL1-like*), G/ACCGC (GCC box, *ERF*), TTAGGC (W-box, *WRKY*), TGACCG (TGACG). Known motifs were identified by TAIR Motiffinder (https://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp) and were present in 1000 bp upstream sequences (only motifs with *p* values equal or less than 1e-03, which are present in 1000 bp upstream sequences of at least 1/4 of all genes in each set are listed). Only genes showing at least a 4-fold up-regulation by Mp were considered, as the full set of 3,136 “only late up” genes did not lead to the identification of conservation motifs satisfying the criteria above (*p* values equal or less than 1e-03; present in 1,000 bp upstream sequences of at least 1/4 of all genes).

ET- and JA-dependent signaling processes have also been implicated in defense against *Mp* in *Medicago truncatula* and *Sesamum indicum*. While *Mp* induced enhanced transcript levels of known JA and ET response genes in sesame plants, treatment of *M. truncatula* with methyl-JA and/or ET enhanced host survival after *Mp* infections. Consistent with a role for ET- and JA-signaling in *Mp* immunity, we also observed up-regulation of numerous Arabidopsis genes related to these processes in *Mp*-infected Col-0 plants. These included genes of various *ERF* (Ethylene Response Factor) transcription factors, JA-responsive JAZ transcriptional regulators as well as ET or JA biosynthetic enzymes. We further found promoter motifs related to known JA- and ET-response elements to be substantially enriched in *Mp*-responsive Arabidopsis genes. Given that *Mp* is considered to exhibit a mostly necrotrophic lifestyle, it is not surprising that Arabidopsis activated ET/JA-dependent immune responses upon infection with this pathogen. Both of these stress hormones are known to control plant immune responses against necrotrophic pathogens. Our results served as critical “proof-of-concept” demonstrating that the new model pathosystem and defense assays we developed allow for the discovery of Arabidopsis processes suppressing *Mp* infections. Furthermore, our results provided an opportunity to use an Arabidopsis line with elevated levels of susceptibility for a comparative transcriptomics study to identify gene expression changes associated with host defense reactions against *Mp*.

Surprisingly, the *Mp*-responsive Arabidopsis transcriptome exhibited substantial overlap with gene sets known to be inducible by SA. Consistent with this, we also observed strong enrichment of promoter motifs related to binding sites of SA-associated TFs in *Mp*-responsive gene sets. Our *Mp* defense assays further confirmed a role of SA signaling in host immunity against this pathogen. Given the predominantly necrotrophic life-style of *Mp*, this was somewhat unexpected, as SA is known to control immune responses against biotrophic pathogens. However, Chowdhury and co-workers recently proposed that in interactions with sesame, *Mp*...
employs a short biotrophic attack strategy prior to switching to a primarily necrotrophic phase. Based on 
* Mp* marker gene expression and morphological features of *M*. *hyphae* between 24 h and 38 h after infection of 
*Arabidopsis*, *Mp* seems to transition from a biotrophic phase to a necrotrophic phase. This “biotrophy-necrotrophy 
switch” (*BNS*) is accompanied by a change of physiological, biochemical and transcriptional responses of the 
host. Most importantly, BNS is associated with a transition from typical SA- to ET/IA-response gene expression 
in sesame. Our results are consistent with the observations and conclusions made by Chowdhury and coworkers.

When interpreting the timing of transcriptional changes in pathogen-infected plants, it is important to con-
sider that infection events do not occur synchronously in all cells/tissue areas of the host. At any time point, only 
the “average” of multiple asynchronized events can be monitored. Thus, the apparent co-occurrence of SA and 
ET/IA-responsive gene expression at the two time points (24 hpc and 48 hpc) studied here may, in fact, reflect 
two successive defense gene expression states in the host. While each individual Arabidopsis root cell may be 
engaged in either pre-BNS or post-BNS response activities, the tested tissue as a whole may represent a mixture 
of response states. Chowdhury and co-workers made similar observations. High resolution time course studies, 
possibly monitoring gene expression events in individual Arabidopsis root cells, may have to be employed in 
the future to determine if an early pre-BNS phase in *Mp* is countered by SA-mediated host defenses and if later 
post-BNS growth of *Mp* induces ET/IA-dependent immunity in Arabidopsis roots.

Crop diseases caused by *Mp* are typically associated with drought and heat stress15. Thus, the induction of 
drought- or heat-tolerance mediating plant stress responses may be linked to immune responses against *Mp*. We 
indeed observed *Mp*-induced up-regulation of numerous Arabidopsis genes associated with drought and heat tol-
erance, such as genes encoding several DREB (Drought-Response Element Binding Protein)-type transcription 
factors, drought tolerance associated LEA (Late Embryogenesis Abundant) proteins, as well as various heat shock 
transcription factors and heat shock proteins, in the RNA-seq data. Plant tolerance to drought stress is partially 
controlled by the phytohormone abscisic acid (ABA). Among genes up-regulated by *Mp* in Col-0 were genes 
encoding the ABA-responsive basic helix loop helix transcription factor BHLH17, the ABA-responsive protein 
ABR and AFP4 (a negative regulator of ABA-responses). Future reverse genetic studies may be performed to test 
if ABA-signaling or other known abiotic stress response mechanisms affect the outcome of *Mp*-Arabidopsis inter-
actions. In any case, our results point toward a complex interplay of multiple plant hormone-controlled signaling 
processes in immunity against *Mp*.

Having established the Arabidopsis-*Mp* interaction as a lab model will allow for faster progress in uncovering 
plant defenses against this detrimental pathogen. In this study, advantages of the Arabidopsis system became 
obvious. An abundance of well-characterized mutants with defined defects in signaling pathways enabling tar-
geting of certain candidate processes (e.g. ET- and JA-signaling) by reverse genetic analyses. The existence of a 
well-annotated Arabidopsis genome sequence allowed for extraction of detailed information from the RNA-seq 
based transcriptomics experiments. Furthermore, a wealth of existing RNA-seq and microarray data was available 
for a wide variety of biological conditions in Arabidopsis for comparison to our results.

Next steps in the use of the new model phyto-pathosystem will include systematic testing of candidate genes 
identified by the RNA-seq study for their contribution to *Mp* defense responses using existing sequence-indexed 
*T*-DNA mutant collections18-21, which collectively cover almost 100% of Arabidopsis genes. The same reverse 
genetics resources can be used to test conserved QTL candidate loci potentially protecting crop species against 
*Mp*. Comprehensive collections of natural Arabidopsis accessions (ecotypes) can be tested against a panel of 
different *Mp* isolates to identify race-specific interactions with differential outcomes. Existing SNP resources 
for many Arabidopsis accessions or recombinant inbred lines31 can be used to map loci responsible for the 
observed differential effects. Finally, Arabidopsis has proven to be an excellent system for high throughput appli-
cations, such as forward genetic screens of large populations of randomly mutagenized individuals44 or chemical 
screens69,85. Mutations or chemicals, respectively, affecting the outcome of plant-*Mp* interactions, can be identified 
this way. We have begun some of these approaches.

Although artificial, the agar plate-based Arabidopsis root assays provided conditions for *Mp* to engage in its 
natural mode of host infection, as microsclerotia formed hyphae, which penetrated dermal root tissues resulting 
in colonization (as seen in soybean19, sesame19, and *M. truncatula*76) and the formation of new microsclerotia 
within the host. Thus, molecular host responses identified in these assays are likely to reflect authentic processes 
occurring during plant-*Mp* interactions under natural conditions. Studying the plant-pathogen interaction on 
agar plates removed interference from unknown structures, compounds or organisms present in autoclaved 
soil that could provide additional variables affecting plant responses or pathogen behavior. The *Mp*-Arabidopsis 
experimental system established here should allow researchers to make great progress regarding the identification 
of key genes affecting the outcome of plant-*Mp* interactions. Knowledge of such genes in Arabidopsis and their 
orthologs in crop species will facilitate the design of new molecular markers for precise marker-based breeding 
approaches in economically important plants. New synthetic elicitors may also be identified that specifically acti-
uate plant immune responses active against *Mp* and which could serve as leads for the development of new pes-
ticide alternatives. Beyond benefiting agriculture directly, discoveries made using our experimental pathosystem 
may allow for the gain of deeper insight into immune responses against *Mp*. The potential interplay of various 
hormone-pathways, which is involved in controlling immunity against *Mp*, is a particularly appealing subject that 
can be approached using our agar-plate-based *Mp*/Arabidopsis infection assays.

**Methods**

**Plant and pathogen growth conditions.** The Arabidopsis double mutant line *ein2-1/jar1-1* has been 
described86. All mutants are in the Arabidopsis ecotype Columbia (Col-0) background: *ein2-1*87, *ein3-1/eil1-1*88, 
.jar1-1*89, *jin1*90, *pad4*91,92, *sid2-2*93, *ein2-1/sid2-2*94, *npr1-1*35,95,96, NahG53, *wrky70*180,180,181* and *wrky70/54*186. Growth 
room conditions were under fluorescent lights (16 h of light/8 h of dark, 23°C, 100 μE m−2 s−1). Seeds were sur-
face-sterilized in 70% ethanol and a 0.02% Triton X, 20% bleach solution, for three and ten minutes, respectively,
followed by sterile water rinses. Seeds were then plated on solid media containing ½ MS (Murashige and Skoog), 0.05% MES, 0.25% sucrose and 0.87% agar; pH was 5.7 prior to the addition of agar. All ½ MS-agar plates contained approximately 25 ml of media. Plated seeds were stratified for two days (or six days for accessions other than Col-0) at 4°C before plates were positioned vertically under growth room conditions for 10 days. Mp-infected roots were placed in acid fuchsia stain99 5 dpc for 18 hours followed by destaining in water.

Mp (originally isolated from UCR Ag Operations Field 11 by members of Philip Roberts’ laboratory89) was propagated by adding a plug to potato dextrose agar (BD DifcoTM, http://us.vwr.com) containing plates (10 ml per plate), incubating at 34°C for 10 days and then allowed to dry at RT for at least four weeks.

**Mp infection assays.** Mp infection plates were created by adding Mp inoculum (approximately 2000 microsclerotia in 2–3 ml media for microsclerotia counting and RNA-seq roots and twice the amount for the shoot disease index assay) to plates, placing plates in a dark incubator at 34°C for three days and then transferring the plates to growth room conditions for an additional three days. The media used to pour plates and create inoculum contained ½ MS (Murashige and Skoog), 0.05% MES, 0.25% sucrose and 0.87% agar; pH was 5.7 prior to the addition of agar. Dried Mp propagation plate contents were ground in 10–20 ml sterile water with mortar and pestle, added to media and counted in 100μl drops on slides until the desired concentration was reached.

For the microsclerotia density assay, 10 day-old seedlings were gently transferred to Mp infection plates, with 12–14 plants per plate, and these plates were placed vertically in growth room conditions for four to five days. To arrest Mp development, 20–30 ml 75% EtOH was added to each plate. Microsclerotia were counted under bright field microscopy and root lengths were measured using ImageJ100. Magnified images were taken using a Leica DM/LB2 (Leica, Wetzlar, Germany) microscope equipped with an RT colour SPOT camera. At least three biological replicates were conducted with a minimum of three plates per line. Statistical significance relative to Col-0 was determined by Student's t-test, p < 0.05.

For the Mp-Arabidopsis shoot disease index, 10 day-old seedlings were gently transferred to Mp infection plates, with two lines per plate, 10 plants per line (n = 30), and these plates were placed horizontally in growth room conditions for two weeks. Images were taken of whole plates throughout the infection period to monitor progress of disease symptoms in plants. Individual plants were assessed according to a disease index scoring system of “0”, for healthy plants, through “5”, for dead plants (Fig. 3B). At least three biological replicates were conducted and significance relative to Col-0 was determined using the Wilcoxon Rank Sum test for two independent samples by Mathcracker on https://mathcracker.com/wilcoxon-rank-sum#results.

**Transcriptome profiling by mRNA-seq.** For RNA-seq, 10 day-old seedlings were transferred to agar plates without Mp or to Mp infection plates, with 12–14 plants per plate, and these plates were placed vertically in growth room conditions for 24 h or 48 h. Plant roots were separated from shoot tissue using a blade and flash frozen in liquid nitrogen at 24 h or 48 h post transfer. Total RNA was isolated from roots using TRIzol (Invitrogen101, http://www.thermofisher.com). RNA was processed (74204 QIAGEN, http://www.qiagen.com, and AM1907 Invitrogen102, http://www.thermofisher.com) and libraries were prepared with the NEBNext Ultra Directional RNA Library Prep Kit for Illumina by following the manufacturer’s instruction (E7490S, E7335S, E7420S, New England Biolabs, http://www.neb.com). Root tissues were separately analyzed for each line (Col-0 or ein2-1/jar1-1), treatment (+/− Mp) and time point (24 h or 48 h). Two independent biological replicates, with three technical replicates for each experimental condition, were performed.

Libraries were pooled and sequenced on an Illumina NextSeq. 500 (Illumina, San Diego, CA, USA) platform at the UCR Genomics Core Facility. Reads that passed Illumina’s quality control filters were further processed. The quality of sequencing reads was assessed using FastQC v 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Unique reads were mapped to the Arabidopsis genome (TAIR10) using STAR v 2.5.3a101 with default settings and a known splice site file, built from Araport annotation file v11. Reads in gene regions were counted using featureCounts103. The expression fold-change of each gene was calculated using the R package DESeq. 2 v1.14.103 with the threshold for differentially expressed genes set to

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value = c
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The MA-plots were generated with the function plotMA in DESeq. 2 and illustrate log-fold change (M-values, i.e. the log of the ratio of level counts for each gene between two samples) against the log-average (A-values, i.e. the average level counts for each gene across the two samples). They showed the log fold changes attributable to a given variable over the mean of normalized counts for the compared samples. Differentially expressed gene points were red if the adjusted p value was less than 0.01. Genes with similar expression levels in two samples appeared around the horizontal line y = 0. Data comparison AGIs can be found in Supplementary Tables S3–S12.

Comparisons between experimental gene sets and sets of genes responding to other stimuli were done using Venny 2.1, (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Supplementary Table S25 groups I–XII GO terminology was compiled at http://go.pantherdb.org/ with the annotation version GO Ontology database, released 2018-04-04, analysis type: PANTHER Overrepresentation Test (released 20171205), test type GO biological process complete with Fisher’s Exact FDR multiple test correction displaying only results with a false discovery rate < 0.05. Supplementary Table S25 groups I–XII AGIs are listed in Supplementary Tables S13–S24.

**Real-time PCR quantification of Mp biomass.** Arabidopsis roots were harvested 48 hpc with Mp for genomic DNA extraction. Genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method104, 1% PVP (chloroform/isoamylalcohol) was added in the 2% CTAB extraction buffer prior to use. After RNase A digestion (19101 QIAGEN http://www.qiagen.com), 20ng of genomic DNA were used for qPCR amplification using the CFX Connect detection system (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad). Two pairs of species-specific primers were used; for Mp DNA amplification: MpSyk-F 3′- ATCCGTGCGGACTGTCCAG-3′ and MpSyk-R 5′- CTGTCCGGAGAAACCGAGAC-3′; for
Arabidopsis DNA amplification: AtSK11-5' - CTTATCGGATTTCTCATGTTTGCC-3' and AtSK11-R 5' - GAGCTCCTGTATTTAACTGTAGACATACC-3'. Melt curve analysis was performed following 40 cycles of amplification with the annealing temperature at 60 °C. The ratios of Mp and Arabidopsis genomic DNA were calculated by the standard curve method. Serial dilutions of Mp and Arabidopsis genomic DNAs were used for standard curve generation (Supplementary Fig. S2). The relative amounts of Mp and Arabidopsis genomic DNA were calculated by normalizing Mp MpSyk31 to Arabidopsis AtSK11 measured by qPCR as described in Gachon & Sandrean, 2004.32

Accession numbers. The Gene Expression Omnibus (GEO) accession number for RNA-seq data reported in this study is GSE127574.

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Author contributions
M.M.S., T.E. and P.R. designed the research. M.M.S., Y.L., M.S. and N.A. performed experiments. T.T. and Y.L. analyzed data. T.E. and M.M.S. interpreted data and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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