Medicago truncatula symbiosis mutants affected in the interaction with a biotrophic root pathogen

Understanding how plants balance between enabling microbial symbionts and fending off pathogens has direct implications both for basic plant biology and optimal use of crop plants in agriculture. The degree to which the processes associated with these two types of interactions overlap is poorly known. Recent studies revealed that symbiotic and pathogenic filamentous microbes require common plant genetic elements to establish colonisation (Wang *et al.*, 2012; Rey *et al.*, 2013), supporting the long-held view that plants have evolved the ability to accommodate microbes (Parniske, 2000) and that pathogens have exploited these pathways. However, the extent to which plant genes implicated in fungal or bacterial symbioses are involved in interactions with biotrophic pathogens is unknown and research has been hampered by the lack of suitable common host experimental systems.

P. palmivora, a root-infecting oomycete, is an aggressive biotrophic pathogen of *M. truncatula*, a model legume plant that is widely used in symbiosis research. Expression of fluorescent proteins in *P. palmivora* permits visualisation of infection structures in *M. truncatula* roots. During its initial biotrophic colonisation of *M. truncatula* roots which lasts until about 48 hours post infection (hpi), *P. palmivora* zoospores expressing tdtomato red fluorescent protein (isolate AJ-td) germinate and form appressoria to penetrate the epidermis followed by rapid colonisation of the root cortex apoplast and projection of short specialised hyphae, termed haustoria into plant cells (Fig. 1a). *P. palmivora* infection is accompanied by visible disease development in *M. truncatula* seedlings consisting of translucent tissue at the root tip at two days post inoculation (dpi) and tissue browning in upper parts of the root at three dpi. (Fig. 1b, see also supplementary material). Concomitant with a switch to a nectrophic lifestyle, the pathogen enters the vasculature (Fig. 1a).

Seven symbiosis genes are implicated in *P. palmivora* disease development

In this study, we took advantage of a newly established quantitative *Phytophthora palmivora-Medicago truncatula* system to assess the extent to which mutants

perturbed in colonisation by arbuscular mycorrhiza fungi (AM fungi) and/or bacterial root nodule symbiosis are affected in the early/biotrophic stages of oomycete pathogenesis (Table S1). We devised and implemented a high throughput seedling infection assay and applied it to 19 *M. truncatula* lines mutated in 14 genes (for details see Methods S1, for explanation of gene abbreviations see Table S1). We measured both the overall root length and disease development, then plotted them as a ratio (Fig. 1a, c; Fig. S1; Table S2). Of the 14 genes tested, seven (nine alleles) showed an altered response to *P. palmivora* inoculation compared to the wild-type Jemalong A17. Mutants in *RAM2* and *NIP/LATD* showed enhanced resistance whereas mutants in five genes, *NFP*, *LYK3*, *ERN*, *EFD*, and *LIN*, all of which are impaired in the interaction with nitrogen fixing rhizobia displayed enhanced susceptibility. Expression levels of two defense response genes in *M. truncatula* mutants with altered disease symptoms were not overall significantly different from levels observed during infection of wildtype A17 seedlings (Fig. S2). This suggests that observed differences in disease extent are not attributable to altered defense responses in these mutants. These findings reveal a significant overlap between processes that define symbiosis and disease in *M. truncatula* roots. However, the remaining *M. truncatula* mutants unaltered in *P. palmivora* disease development include the common symbiotic signalling pathway (CSSP) mutants *dmi1*, *dmi2* and *dmi3*, suggesting that the CSSP is not a major modulator of susceptibility to *P. palmivora* in *M. truncatula.*

The mutants *nfp-1*, *nfp-2* and *hcl-2* encode defective NFP and LYK3 LysM domain receptor-like kinases (LysM-RLKs) (Table S1). NFP and LYK3 are required for plant responses to lipochitooligosaccharides (LCOs) from root nodule bacteria and mutants in both genes are impaired in nodule formation (Arrighi *et al.*, 2006; Smit *et al.*, 2007). Furthermore, *nfp-1* is affected in transcriptional responses to fungal and bacterial LCOs (Czaja *et al.*, 2012). Interestingly, *nfp-1, nfp-2* and the *lyk3* mutant *hcl-2* showed enhanced disease symptoms during *P. palmivora* infection (Fig. 1c). Rey *et al.* previously reported that *nfp mutants* displayed enhanced susceptibility to the *P. palmivora* unrelated oomycete *Aphanomyces euteiches* and the fungal pathogen *Colletotrichum trifolii* (Rey *et al.*, 2013). Our data further strengthens a role for NFP as negative regulator in plant interactions with a biotrophic filamentous pathogen. Given that both, NFP and LYK3 are involved in bacterial LCO perception,

we tested whether *lyk3/hcl* mutants are also displaying enhanced susceptibility to *P. palmivora*. We found, that *hcl-2* but not *hcl-1* was altered in disease development. The *hcl-1* allele harbours a missense mutation in the conserved kinase I motif presumably rendering it non-functional. Conversely, *hcl-2* carries a splice site mutation giving rise to transcripts potentially encoding severely truncated LYK3 proteins or a full length protein with significant part of LysM 3 domain deleted (Smit *et al.*, 2007). It is possible that truncated LYK3 proteins specifically interfere with defense signalling elements involved in *P. palmivora* perception.

The U-Box/WD40 LIN protein functions during rhizobial colonization and abortion of this process in *lin-2* leads to a suppression of nodule development (Kuppusamy *et al.*, 2004; Kiss *et al.*, 2009). Identification of LIN targets and their functional tests in *P. palmivora* interactions should aid future dissection of common and specific regulatory nodes for root nodule development and pathogen colonisation. Taken together, our results thus highlight the need for understanding which are the common mechanisms involved in symbiosis and disease development.

Symbiosis and disease responses to *P. palmivora* are integrated into two common transcription factor targets via different regulatory nodes

We studied transcription factors and their target genes to address whether the regulatory networks identified in symbiosis also apply for *P. palmivora* disease development. Expression of symbiosis genes in part is regulated by GRAS-type transcription factors such as *RAM1* and *NSP1* and *NSP2* which act as heterodimers. *RAM1/NSP2* (Gobbato *et al.*, 2012) regulate *RAM2* expression and NSP1/NSP2 activate *ERN1* (Cerri *et al.*, 2012). Mutations in *RAM1, NSP1, NSP2* or *ERNs* are all impaired in either mycorrhiza or nitrogen fixing symbiosis (see references in Table S1). Here we confirmed requirement of *RAM2* for *P. palmivora* infection. Moreover, we found that *ERN1* negatively regulates *P. palmivora* disease development. However, mutations in the *RAM2* and *ERN1* expression regulating transcription factors *RAM1* and *NSP1* are dispensable for *P. palmivora* infection (Fig. 1c). This suggests that symbiosis and disease responses to *P. palmivora* may be integrated into common elements via different regulatory nodes resulting in opposite outcomes.

Taken together, the disease phenotypes we report here should help understanding the biological processes perturbed in symbiosis mutants.

Mutants reveal an overlap between plant development and disease resistance processes

Phytophthora, a genus of destructive plant pathogens of global importance to agriculture, is formed primarily of species that cause disease on plant roots. Yet only a few plant mutants affected in response to root infection by *Phytophthora* pathogens have been reported. Here, we implicate seven genes in disease development to *P. palmivora* in roots and gained new insight on the host genetic requirement for limiting pathogen infection. Two mutants showed enhanced resistance to *P. palmivora* and ram2-1 has previously been demonstrated to be more resistant to *P. palmivora* as well as *Aphanomyces euteiches* (Wang et al, 2012,Gobbato *et al.*, 2013). However, both *ram2-1* and *latd* mutants still display a limited degree of colonisation, likely attributable to the ability of *Phytophthora* species to utilise alternative infection routes which do not require appressorium mediated cell penetration such as penetration through middle lamellae of anticlinal host cell walls (Hardham, 2001) or through wounds (Drenth & Guest, 2004).

We assessed whether *ram2-1* and *latd* are impaired in haustorium formation using *P. palmivora* strains expressing fluorescent proteins. We detected presence of haustoria in both, *latd* and *ram2-1* (Fig. 1d, Fig. S4) as well as in all other tested mutants (Fig. S3). Notably, *ram2-1* is strongly impaired in arbuscule formation (Wang *et al.*, 2012). We speculate that both, *latd* and *ram2-1* are impaired in entering the root resulting in a lower overall infection efficiency. *RAM2* has been reported to be impaired in production of cutin monomers required for appressorium formation as well as seed coat processes (Wang *et al.*, 2012).

The mechanistic basis for resistance in *latd* remains to be studied but possibly points to a role of plant hormone and reactive oxygen species (ROS) regulated processes in the interaction between *P. palmivora* and *M. truncatula*. ROS levels are elevated in *latd* mutants (Zhang *et al.*, 2014). The *LATD* gene encodes a predicted NRT1(PTR) transporter (Yendrek *et al.*, 2010), and the *latd* mutant (W341STOP) has

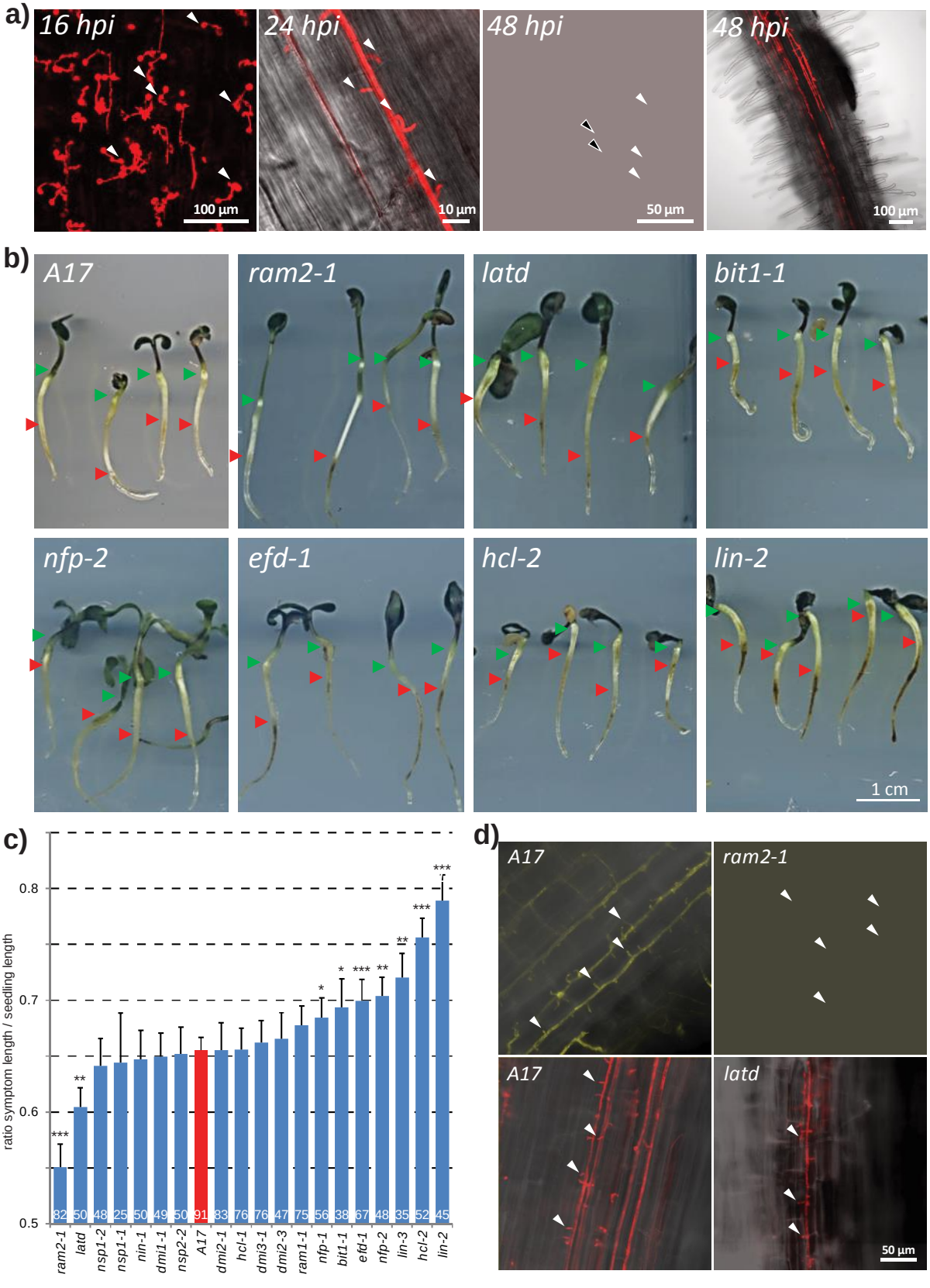
defects in root architecture including root hair defects and lateral roots do not fully differentiate. *Mtlatd* also has defective nodules (Bright *et al.*, 2005). Mutant *latd* plants have wild-type levels of endogenous ABA, but exhibit reduced sensitivity to the effect of ABA on stimulation of both stomatal closure and inhibition of seed germination (Liang *et al.*, 2007). Future experiments should address whether high levels of ABA can complement the *P. palmivora* disease symptom phenotype in a similar manner to ABA treatment reversed ROS accumulation (Zhang *et al.*, 2014).

EFD is another modulator of hormone signalling, that was found to be important for *P. palmivora* infection. *EFD* is an ERF transcription factor which negatively regulates nodule development through interference with cytokinin signalling and mutants result in a higher number of nodules defective in nitrogen fixation (Vernié *et al.*, 2008). Likewise, we observed extended disease symptom length with *P. palmivora* in *efd-1* plants. Given, that *efd-1* is not affected in colonisation by AM fungi, we hypothesize that *EFD* modulates symptom development rather than early colonisation by *P. palmivora*. Considering that *EFD* affects a cytokinin signalling response regulator, future work should assess the importance of cytokinin signalling on root colonisation by *P. palmivora*. Taken together, our data implies a role for the plant hormones ABA and cytokinin in disease development.

Conclusions

Our finding, that seven out of 14 genes implicated in symbiosis also impact *P. palmivora* disease development highlights that several plant symbiosis genes are involved in interactions with biotrophic pathogens. This is consistent with the long-held view that plants have evolved the ability to accommodate microbes and that pathogens have exploited these pathways (Parniske, 2000). However, new questions arise from this work. Five genes exert opposing roles in symbiosis vs. disease development. Perhaps, plants utilise these genes to balance between defence and symbiosis. Surprisingly, mainly nitrogen fixation mutants but not mycorrhiza mutants were affected in *P. palmivora* infection. This might highlight an overlap in general cellular and developmental processes which have not been picked up by the slower and often less sensitive AM fungus colonisation assays. Also, several mutants used in this study remain to be thoroughly characterized for their

mycorrhizal phenotype. It is conceivable, that testing of all available symbiosis mutants with biotrophic pathogens will further our understanding on the overlap of molecular mechanisms underpinning symbiosis and disease development. Assessing their role in disease development in plant species which only support one type of symbiosis, mycorrhiza or nitrogen fixing symbiosis could enable further disentanglement of molecular pathways. Genetic and biochemical characterisation of proteins with opposing outcomes will provide inroads to engineering disease resistant plants which retain symbiosis capabilities.


a)
16 hpi
100 µm
24 hpi
10 µm
48 hpi
50 µm
48 hpi
100 µm
b)
A17
ram2-1
latd
bit1-1
nfp-2
efd-1
hcl-2
lin-2
1 cm
c)
ratio symptom length / seedling length
0.8
0.7
0.6
0.5
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82 50 48 25 50 49 50 91 83 76 76 47 75 56 38 67 48 35 52 45
ram2-1
latd
nsp1-2
nsp1-1
nin-1
dmi1-1
nsp2-2
A17
dmi2-1
hcl-1
dmi3-1
dmi2-3
ram1-1
nfp-1
bit1-1
efd-1
nfp-2
lin-3
hcl-2
lin-2
d)
A17
ram2-1
A17
latd
50 µm

Figure Legends

Figure 1. *Medicago truncatula* mutants with altered symbiotic interactions display differential degrees of resistance to the filamentous pathogen *Phytophthora palmivora*.

(a) Biotrophic infection stages of *P. palmivora* AJ-td in *M. truncatula* Jemalong A17 roots. At 16 hours post inoculation (hpi) spores and appressoria (arrowheads) are visible at the epidermis. At 24 hpi apoplastic hyphae and haustoria (arrowheads) in root cortex cells are visible. At 48 hpi the root is extensively colonised with biotrophic hyphae running parallel to the root axis and forming haustoria in the cortex (filled arrowheads). Hyphae entering the central cylinder can also be observed (empty arrowheads).

(b) Seedling zoospore inoculation phenotypes 3 days post inoculation (dpi). Extent of symptoms (translucent dead tissue and browning) from root tip (red arrowhead) and overall assessed seedling size (green arrowhead) are indicated. Magnification of all images is identical.

(c) Disease symptom extent (symptom length/seedling length) of *M. truncatula* seedlings inoculated with the root pathogen *P. palmivora* scored 3 dpi. All experiments involved at least three inoculation repeats with at least 10 plants for each genotype. Number of assessed seedlings per genotype is displayed at the base of the bars. Bars represent average with standard error, Asterisks represent probability values following statistical t-test * $p<0.05$, ** $p<0.01$, *** $p<0.001$

(d) Confocal imaging stacks of *M.truncatula* roots colonised with LILI-YKDEL (upper row) imaged at 24 hpi and AJ-red (lower row) imaged at 42 hpi reveal the presence of haustoria (arrowheads) in wildtype *M. truncatula* Jemalong A17 (A17) as well as in the mutants *ram2-1* and *latd.* Magnification of all images is identical. Higher magnification images can be found in Fig. S4.

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Supplementary information

Figure S1. Average root length and average length of symptomatic tissues upon infection by *P. palmivora* 2 days post inoculation.

Figure S2. Induction of defence associated genes in mutants and A17 wild type (WT) upon infection by *P. palmivora*

Figure S3. *Medicago truncatula* symbiosis mutants permit haustorium formation by *Phytophthora palmivora*

Figure S4. Magnified single confocal plane images of haustoria

Table S1. Brief description of *M. truncatula* mutants used in this study.

Table S2. Data obtained from disease extent scoring

Methods S1. Biological material, growth conditions, inoculation assays, microscopy and gene expression analysis

Key words

Medicago truncatula, *Phytophthora palmivora,* Symbiosis, Immunity, Haustoria, Root nodules