Use of ecologically-and evolutionary relevant transcriptomic data to infer functions of fungal pathogen gene orthologues essential for limiting fungal stresses caused by interacting host plants and bacteria

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SO Initial idea. Hypotheses generation from data and from literature, co-regulation analysis related to HPI, overall responsible for driving the work forward and methods development. Primary responsible for manuscript writing and the analysis of data. Coordination of manuscript writing and corrections.

O.A. Research concerning MoCpa1 and MoADE4, Manuscript correction

H.L. Research concerning *F. graminearum* NO production. Manuscript correction

Q.L. Analysis of PARP/PARG in the secondary data. Manuscript correction

BOH Research concerning *F. graminearum* NO production and CK2 activity in *F. graminearum* and *M. oryzae* during different HPI normalized for growth. Download and preparation of secondary data. Manuscript correction.

W.T. Research concerning MoCpa1 and MoADE4. Manuscript correction.

Z.W. Research concerning *F. graminearum* NO production, CK2 activity in *F. graminearum* and *M. oryzae* during different HPI normalized for growth, MoCpa1 and MoADE4. Acquisition of financial support. Manuscript correction.

G.L. Research concerning *F. graminearum* NO production, Acquisition of financial support. Manuscript correction.

W.Z. Research concerning *F. graminearum* NO production. Manuscript correction.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 March 2021 doi:10.20944/preprints202012.0773.v2

ABSTRACT

Key genes needed for maintenance and growth for the two pathogens, *Fusarium graminearum* and *Magnaporthe oryzae,* were identified. These are genes that are induced in response to maintenance requirements (stress) and growth requirements. The processes involved are synthesizing arginine, synthesis of DNA-bases, nitric oxide synthesis needing arginine, autophagy, DNA synthesis, and DNA repair. A simplified regulatory network for these key genes for both organisms was constructed as a hypothesis for the work, and procedures previously developed to use sets of downloaded transcriptomic data were used to test hypotheses concerning what time under the course of infection of plants the key genes are expressed. The analysis shows that the transcription efforts (costs) to maintain the fungal cells (maintenance) are high before infection and during early infection. During the following biotrophic stage, maintenance activities drop, followed by a dramatic increase in the necrotrophic stage transition. Finally, in the necrotrophic stage, maintenance is again lower despite the high growth rate that can also cause stress. All identified genes' expressions behaved almost similar with an increased expression in the biotrophy-necrotrophy transition for both fungi except the DNA repair genes PARP/PARG that was not responding or absent (PARG) in the mainly clonal *M. oryzae.* This PARG expression pattern might indicate that *M. oryzae* is more subject to evolution by point mutations than *F. graminearum,* where sexual reproduction is frequent. The potential consequences of this in the development and the accelerated breakage of host species resistance in a Red Queen dynamics scenario are discussed. The analysis demonstrates the possibility of using large transcriptome datasets and co-regulations between key genes to test hypotheses. This technique's advantages complement molecular techniques that employ knockouts and over-expression of target genes to suggest that genes' roles are discussed.

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INTRODUCTION

Growth and maintenance are vital concepts both in cell biology and ecology. Maintenance is all the activities needed to maintain the cell's integrity, including repairing proteins and DNA without making more biomass [1]. Growth is simply the growing of biomass and cells' growth, including all proteins needed for making new cells and maintenance, including the necessary new copies of the genomes [2]. However, the maintenance concept has been questioned since it contains many different processes [3].

Wheat head blight caused by *F. graminearum* Schwabe (teleomorph stage: *Gibberella zeae* (Schwein.) Petch) often results in significant crop losses in grains like wheat and barley [4]. *Magnaporthe oryzae* B.C. Couch (teleomorph *Pyricularia oryzae* Cavara) cause rice blast resulting in yield and economic losses worldwide [5]. Both fungi are studied by many researchers worldwide, and they are both considered model organisms [6,7]. One interesting difference between them is that *F. graminearum* often reproduces sexually [8] while *M. oryzae* is mainly clonal [9]. Genetically, these two *Ascomycete* pathogens are relatively closely related and differ from yeast and Penicillium/aspergillus species. Both fungi belong to the class *Sordariomycetes* but in different orders. *F. graminearum* belongs to *Hypocreales* and *M. oryzae* to *Magnaporthales*. Most genes and gene expression patterns are mirrored in the two species [10]. Both pathogens infect as biotrophs and switch to necrotrophy at a later stage Hours Post Infection (HPI). They are exposed to environmental stresses at the plant surfaces, including possible biotrophic stresses from other organisms. They enter the plant and establishes biotrophic growth inside the plant. The plant defences are low at this time. At about mid-time (HPI), the fungi become detected by the plant's innate immune system that starts attacking the intruders with radical oxygen species (ROS). In response to this the pathogens switch to necrotrophy, killing the host cells and in the case of *F. graminearum* producing the toxic secondary metabolites deoxynivalenol (DON) [4]. At the end of the necrotrophic stage both fungi switch from biomass growth to conidia production emptying the vegetative mycelium of biomass to form conidia that can spread to other plants and infect them. In a previous study we found that the expression of the key autophagy gene ATG8 increases with HPI in both fungi and can be used as indicator for HPI in downloaded expression data from a large number of experiments [10]. In the same study we identified the His2b gene as an indicator of *de novo* DNA synthesis and growth since free histones not bound to DNA are cytotoxic [10,11]. Maintenance expression of a specific gene is defined as the relative growth rate normalized transcript expression of the gene, or in other words, gene expression normalized for DNA synthesis [10].

We have previously studied conserved genes involved in fungal maintenance and growth. As the primary gene regulated during autophagy ATG8 [12], the DNA repair gene PARP [13,14], and recently we have worked with genes involved in the synthesis of DNA bases [15] and the amino acid arginine [16]. Arginine was shown to be used together with oxygen to produce nitric oxide (NO), a ROS produced in fungal innate immunity [17]. Plants trigger NO-production during the transition between biotrophy to necrotrophy, and when the fungus is exposed to bacterial MAMPs (microbial-associated molecular patterns) [17]. Together, these genes fit into a conceptual model for how these conserved genes necessary for growth and maintenance are likely to be differentially expressed during different stages of a plant's plant pathogen colonization. During growth-dominated stages, the purine synthesis genes are mainly used to make new DNA, while arginine synthesis is primarily needed to make new proteins. ATG8 activity is also crucial for growth since growing fast causes a need to recycle misfolded proteins, protein aggregates, and storage lipid droplets through autophagy [10,12,18] (**Fig. 1A**).

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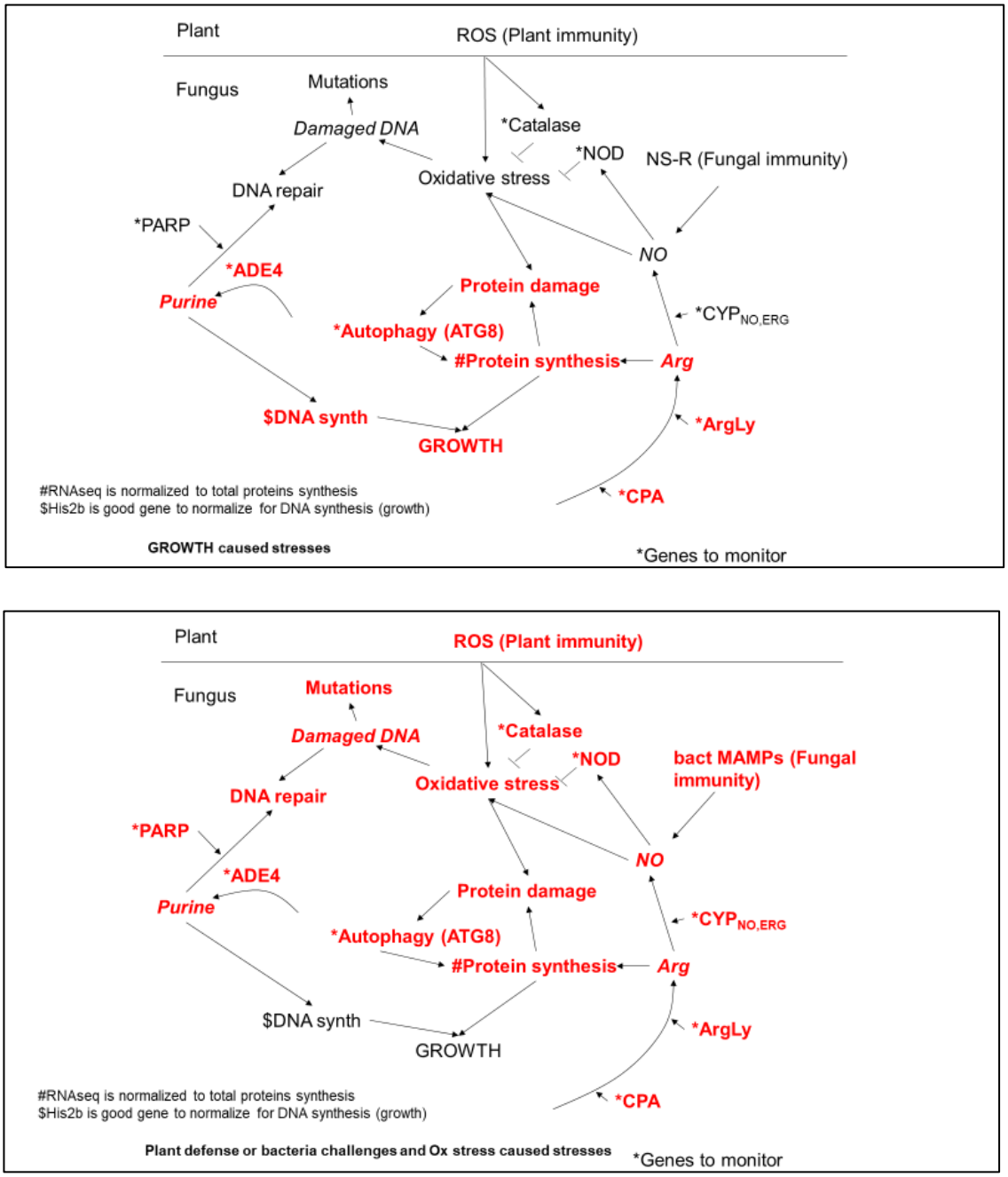

Figure 1. Hypothesized transcriptional activity of identified genes **A**. During biotrophic growth and later stages of necrotrophy, the growth-related network marked red should be most active transcriptionally and reflected by the transcription of the respective gene. **B.** When the fungus is exposed to plant-induced stresses, the stress-related network marked red should be most active. There will be a focus on this network if gene expression is normalized for growth using the His2b gene transcriptional expression [10] to highlight maintenance needed to counteract the plant ROS defences. CPA=MoCPA1 [19] and FgCPA1 (this study), ArgLy=MoArgLy [20] and FgArgLy (this study), CYP(NO,ERG)=FgCYP(NO,ERG) [17] and MoCYP(NO,ERG) (this study), ATG8=MoATG8 [21] and FgATG8 [12], PARP=FgPrp (this study) and MoPrp (this study), ADE4=MoADE4 [15] and FgADE4 (this study), NOD=FgNOD1 and FgNOD2 [17] and MgNOD and (this study), finally catalases Catalase=FgCAT1-5 (this study) and MoCAT1&3 (this study). For more info information on these genes, see **Table 2**.

This hypothesis that the stress weighted network (**Fig. 1B**) should be most active just before penetration and especially in the transition between biotrophy to necrotrophy when plant defences are activated was tested. In contrast, during biotrophic growth and later stages of necrotrophy, the growth-related network (Fig. 1A) should be more active. For *F. graminearum* and *M. oryzae,* we used 64 respective 47 transcriptomes sampled at different times post-infection [10]. The activity of the genes of interest under non-growing conditions short time (<4h) after challenging with purified bacterial MAMPs in a dataset of 113

transcriptomes [17,22] was also tested for *F. graminearum*. Exposure to bacterial MAMPs triggers NO production, and the MAMPs responses should mainly be reflected as an increased response of the stress weighted network (**Fig. 1B**). The stress-weighted network should increase in expression with the expression of the cytochrome p450 gene (CYP(NO,ERG)) that is the gene mainly responsible for the intrinsic NO production with accompanying ROS stress [17].

The data supported the hypothesis, and also, it was found that the PARP gene necessary for DNA repair is expressed very differently in *M. oryzae* and *F. graminearum*. An orthologue for PARG necessary for de-PARylation of the PARP activity is absent in *M. oryzae*. It is suggested to interpret the found difference that this reflects the need for the mainly clonal *M. oryzae* [8,9] to generate variation through mutations to overcome host resistance changes without sexual recombination. Finally, the potential benefits of transcriptomic analyses for suggesting the relative importance of specific gene expressions and roles of genes under relevant natural conditions are discussed

MATERIALS AND METHODS

The procedures are briefly outlined in the Results and Discussion section and mainly comprise plotting of transcript expression data against transcript expression data (RNAseq or Affymetrix microarray data). All data used for this paper are secondary data and have been described in previous articles and are publicly available (**Table 1**). Candidate orthologous genes were identified through protein BLAST at NCBI (**Table 1**).

Table 1. supplemental material available at public websites.

RESOURCE	SOURCE	IDENTIFIER
Deposited Data		
M. oryzae transcriptomic data matrix covering a range of plant infection experiments	[10]	DOI: 10.6084/m9.figshare.7068857
F. graminearum transcriptomic data matrix covering a range of plant infection experiments	[10]	DOI: 10.6084/m9.figshare.7068860
F. graminearum transcriptomic data matrix covering a range of experiments of stationary F. graminearum mycelia in water exposed short times (1,2,4h) to purified bacterial MAMPs	[17] Supplementary data	DOI: https://doi.org/10.1101/2020.07.12.191361

Table 2. Genes analyzed in this paper and how these genes were identified

From published papers or annotated at NCBI		
Fg or Mo genes	Abbr. /ID	**SOURCE**
Fg CYtochrome P450 $CYP_{(NO,ERG)}$	$FgCYP_{(NO,ERG)}$/ FGSG_01000	[17]
Fg Nitric Oxide Dioxygenase1	FgNOD1/ FGSG_00765	[17]
Fg Nitric Oxide Dioxygenase2	FgNOD2/ FGSG_04458	[17]
Fg ATG8	FgATG8/ FGSG_10740	[12]
Mo ATG8	MoATG8/ MGG_01062	[21]
Fg Histone 2b	FgHis2b/ FGSG_ 11626	[10]
Mo Histone 2b	MoHis2b/ MGG_03578	[10]
Mo carbamoyl phosphate synthetase small subunit	MoCPA1/ MGG_01743	[16]
Mo Arg Lyase (ARG4)	MoArgLy/ MGG_17278	[20]
Mo Amido phosphoribosyl transferase	MoADE4/ MGG_04618	[15]
Mo Catalase1	MoCAT1/ MGG_10061	XP_003717445.1
Mo Catalase1	MoCAT3, MGG_06442	XP_003717126.1

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Through BLAST comparison with proteins using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) *Fusarium graminearum* genes				
Organism (Code)	**Annotated gene**	**Orthologue/ID**	**E-value (%ID)**	**Cover-age %**
M. oryzae	MoCPA1 See above	FgCPA1 FGSG_09554	0.0 (79.82)	94
M. oryzae	MoArgLy See above	FgArgLy FGSG_03694	0.0 (70.11)	97
M. oryzae	MoADE4 See above	FGSG_05278 FgADE4	0.0 (65.60)	84
Metarhizium robertsii	MrBI-1 XP_007826493.1|	FgBI1 FGSG_09422	0.0 (88.41)	100
Aspergillus nidulans	PrpA AAQ23182.1	FgPrp FGSG_05924	2e-175 (50.83)	78
Saccharomyces cerevisiae	CatA DAA12096.1	FgCAT1 FGSG_02881	9e-134 (43.27)	91
S. cerevisiae	CatA DAA12096.1	FgCAT2 FGSG_05695	1e-125 (42.31)	95
S. cerevisiae	CatA DAA12096.1	FgCAT3 FGSG_06596	2e-105 (40.00)	86
S. cerevisiae	CatA DAA12096.1	FgCAT4 FGSG_06554	6e-85 (41.97)	67
S. cerevisiae	CatA DAA12096.1	FgCAT5 FGSG_06733	1e-83 (39.34)	69
Fusarium oxysporum	FoPARG1 XP_018241514.1	FgPrg FGSG_09290	0.0 (70.34)	91
***Magnaporthe oryzae* gene (mostly identified through BLAST comparisons with *F. graminearum*)**				
F. graminearum	FgCYP$_{(NO,ERG)}$ See above	MoCYP1$_{(NO,ERG)}$ MGG_04432	0.0 (72.11)	99
F. graminearum	FgCYP$_{(NO,ERG)}$ See above	MoCYP2$_{(NO,ERG)}$ MGG_04628	0.0 (55.78)	94
F. graminearum	FgNOD1 and 2 See above	MoNOD MGG_00198	2e-116 (42.73)	90
Metarhizium robertsii	MrBI-1 XP_007826493.1|	MoBI1* MGG_00198	5e-145 (74.47)	100
Aspergillus fumigatus	PrpA AAQ23182.1	MoPrp MGG_08613	0.0 (48.24)	87

*Annotated as MoBI-4 at NCBI, XP_003720584.1

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Data handling and analyses of linear regression correlations

All transcriptional data was handled and plotted in MS Excel and assembled into figure plates using MS PowerPoint. Regression analyses were performed as Reduced Major Axis (RMA) regression to address random variation in both the x and y variables [23] since measured data for the Log2 expression of two genes were always compared. For these analyses, expression data were transferred to the statistical freeware PAleontological Statistics [24] (PAST: https://palaeo-electronica.org/2001_1/past/issue1_01.htm). The linear regressions parameters were subsequently used to plot fitted lines using M.S. Excel. P values for the null-hypotheses of no correlation, and the probability that slopes for correlations of different gene pairs are different are given in the relevant figure legends.

RESULTS AND DISCUSSION

Since the quality of the used transcriptome dataset is better for *F. graminearum* [10], this dataset was first investigated for the expression of the five key genes (**Fig. 1**) and compared to the expression of the FgATG8 gene as an indicator of HPI [10]. The switch from biotrophy-necrotrophy occurs around LOG2 ATG8 expression values of 4.5-6 as indicated by expression of the TRI4 gene involved in DON production needed for counteracting plant defences [10,17]. All five key genes are upregulated at the shift from biotroph to necrotrophy (**Fig. 2A-E**). All five genes show similar temporal patterns of growth-normalized gene expression (**Fig. 2F-J**), supporting the presented hypothesis (**Fig. 1**). The genes appear essential for maintenance to handle the cellular stresses at low HPIs (LowATG8 expression) and are then upregulated. After that, expression of the genes decreases during biotrophy, followed by a substantial increase during the biotrophy-necrotrophy transition. Finally, all genes' expression decreases again during necrotrophy, tending to a final rise at very late necrotrophy.

The shapes of these growth-normalized expression profiles are, in principle, W-shaped.

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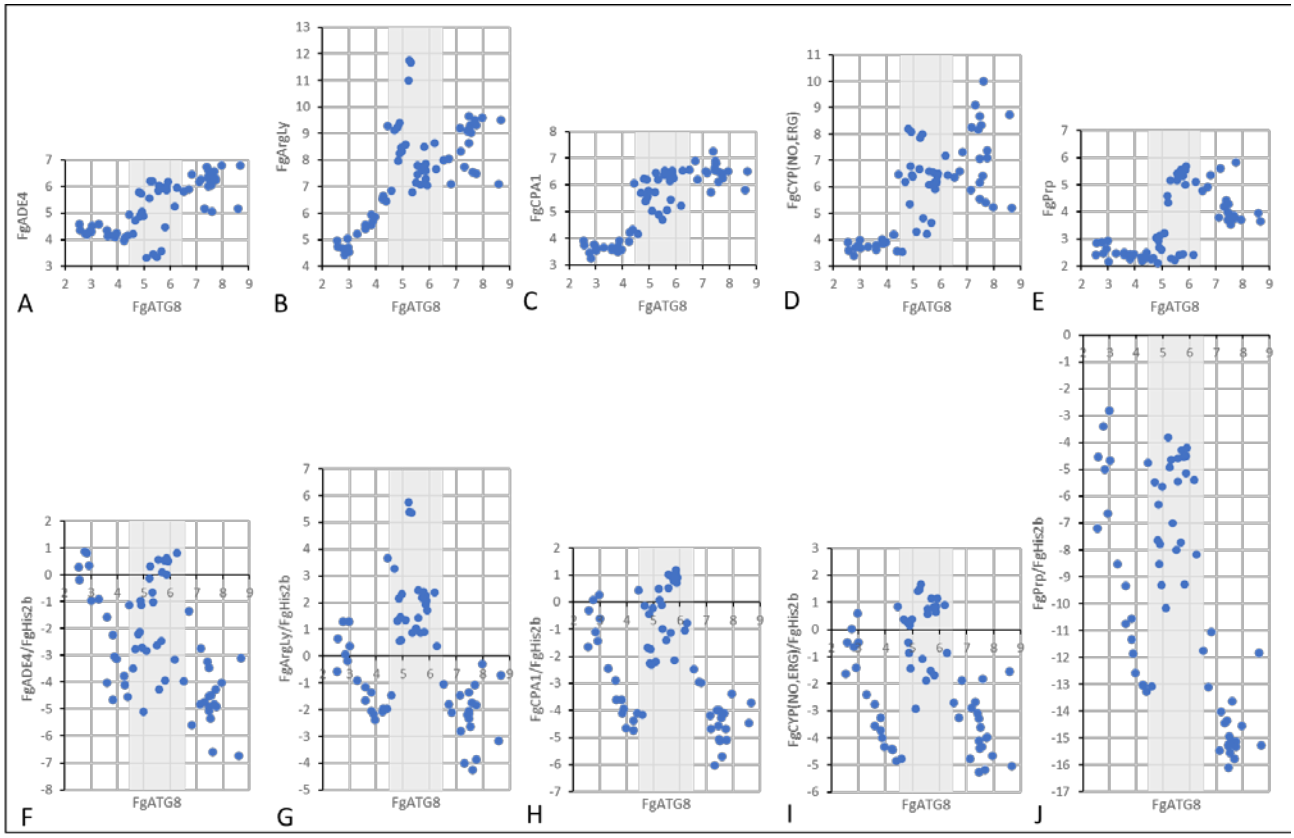

Figure 2. LOG2 expression of key genes compared to the LOG2 expression of the autophagy gene ATG8 is increasingly expressed during plant infection. The transition between biotrophy to necrotrophy occurs at LOG2 ATG8 expression values of 4.5-6. (**A-E**) Total expression (growth + maintenance related). (**F-J**) Maintenance-related, growth-normalized expression using FgHis2b for normalization vs. FgATG8 (HPI). All plots (**A**-J) are shown with equal scaling on both axes to facilitate comparisons. Grey areas mark the biotrophy/necrotrophy transition (Log2FgATG8=4.5-6.5).

For *M. oryzae,* as for *F. graminearum,* an increased transcription was observed for most of the five genes (**Fig. 3A-E**). Growth-normalized FgPrp was strongly upregulated, especially at the transition between biotrophy to necrotrophy, while this was not found for MoPrp (**Fig. 3F-J**). The lack of regulation in *M. oryzae* could indicate that very little ROS stress is experienced by the fungus in the transition between biotrophy to necrotrophy. Alternatively, MoPrp is not functionally regulated in response to DNA damages caused by oxidative stress since MoPrp can have lost both function and regulation.

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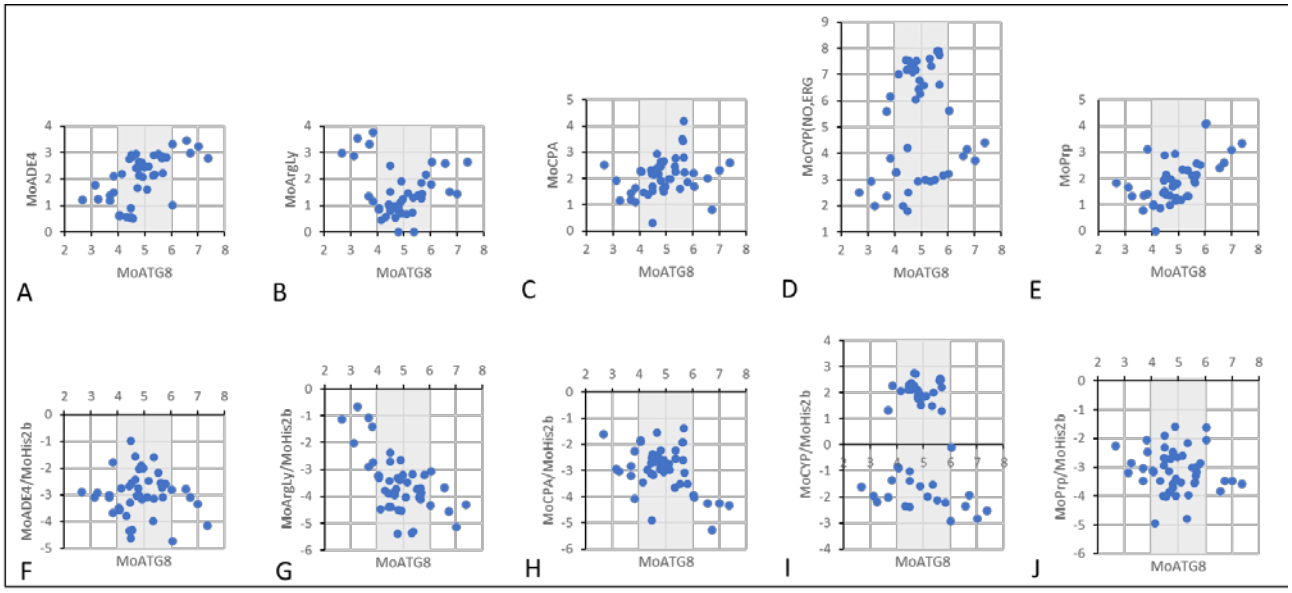

Figure 3. LOG2 expression of key genes compared to the LOG2 expression of the autophagy gene ATG8 is increasingly expressed during plant infection. The transition between biotrophy to necrotrophy occurs at LOG2 ATG8 expression values 4-6. **A-E**. Total expression (growth + maintenance related). **F-J**. Maintenance-related, growth-normalized expression using MoHis2b for normalization vs MoATG8 (HPI). All plots (**A-J**) are shown with equal scaling on both axes to facilitate comparisons. Grey areas mark the judged biotrophy/necrotrophy transition (Log2 MoATG8=4-6).

Oxidative stresses due to hydrogen peroxide or nitric oxide

The relative expression for all catalase (CAT) orthologues in both fungi was investigated to investigate ROS stress. Catalase is needed to counteract intrinsically, and plant-made H_2O_2 nitric oxide dioxygenase (NOD) orthologues are necessary to balance intrinsically and plant-made NO. Five decent candidate orthologues to yeast (*Saccharomyces cerevisiae*) CatA, genes FgCAT1-5 were found for *F. graminearum* (**Table 2**). In *F. graminearum,* there are two nitric oxide dioxygenase genes [17], and two orthologues to these were found in M. oryzae (**Table 2**).

F. graminearum catalase gene responses

All five catalase orthologues are activated in the biotrophy to necrotrophy transition (**Fig. 4**). The activation was pronounced when investigating the growth-normalized expression where the expression profiles for all genes resembled the expression profiles for the PARP orthologue (FgPrp) (**Fig. 2E and J**).

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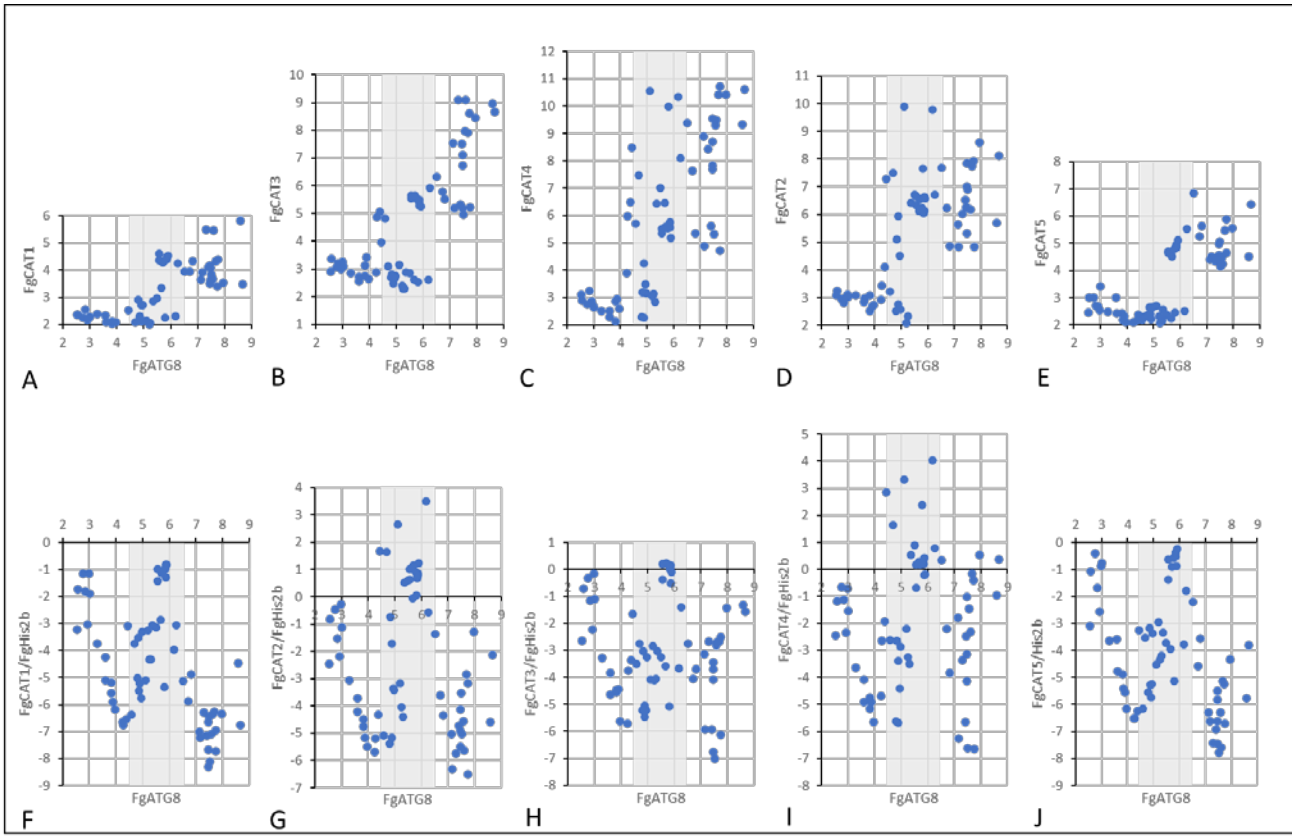

Figure 4 Expression of catalase orthologues in *Fusarium graminearum*. **A-E.** Expression vs FgATG8 (HPI). **F-J.** Growth-normalized expression using FgHis2b for normalization vs. FgATG8 (HPI). All plots (**A-J**) are shown with equal scale tick marks on both axes to facilitate comparisons. Grey areas mark the judged biotrophy/necrotrophy transition (Log2FgATG8=4.5-6.5).

If these genes' expression responds to oxidative stresses that cause DNA damage, they should be correlated with FgPrp expression, and when both gene expressions are growth-normalized, they are (**Fig. 5A-E**).

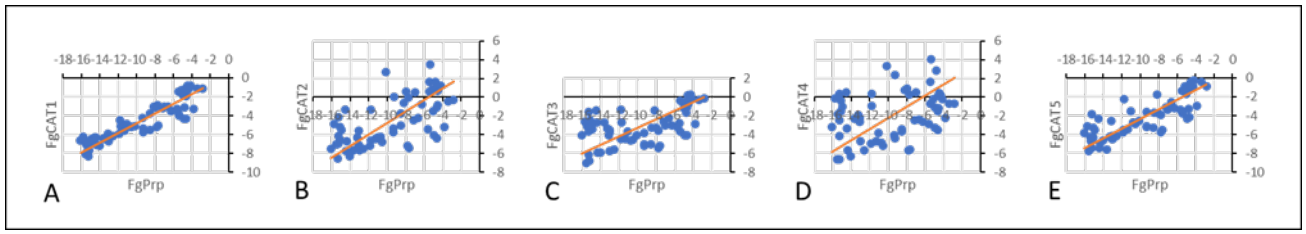

Figure 5.A-E. Catalase homologs vs FgPrp in *F. graminearum* both normalized for growth. There are strong correlations between the FgCATs and FgPrp, as expected if all FgCATs and FgPrP help the fungus against H_2O_2. All plots (**A-E**) are growth-normalized and shown with equal scaling on both axes to facilitate comparisons. RMA regressions are shown as red lines. P for not correlated where FgCAT1=1.32E-26, FgCAT2=9.97E-10, FgCAT3=1.62E-06, FgCAT4=2.24E-03, FgCAT5=6.06E-17. The slopes of all correlations were not significantly different since, in pairwise comparisons, they showed overlapping 95% confidence intervals for their calculated slopes.

M. oryzae catalase gene responses

Two catalase gene orthologues are annotated for *M. oryzae* (**Table 2**). These were also only one of the catalase genes with BLAST similarities to yeast CatA. Of these, only MoCAT1 responds strongly by an upregulation in the transition between biotrophy to necrotrophy (**Fig. 6A**) and appears to stay high also growth-normalized in the whole necrotrophic stage at high MoATG8 expression (**Fig. 6A-iii**).

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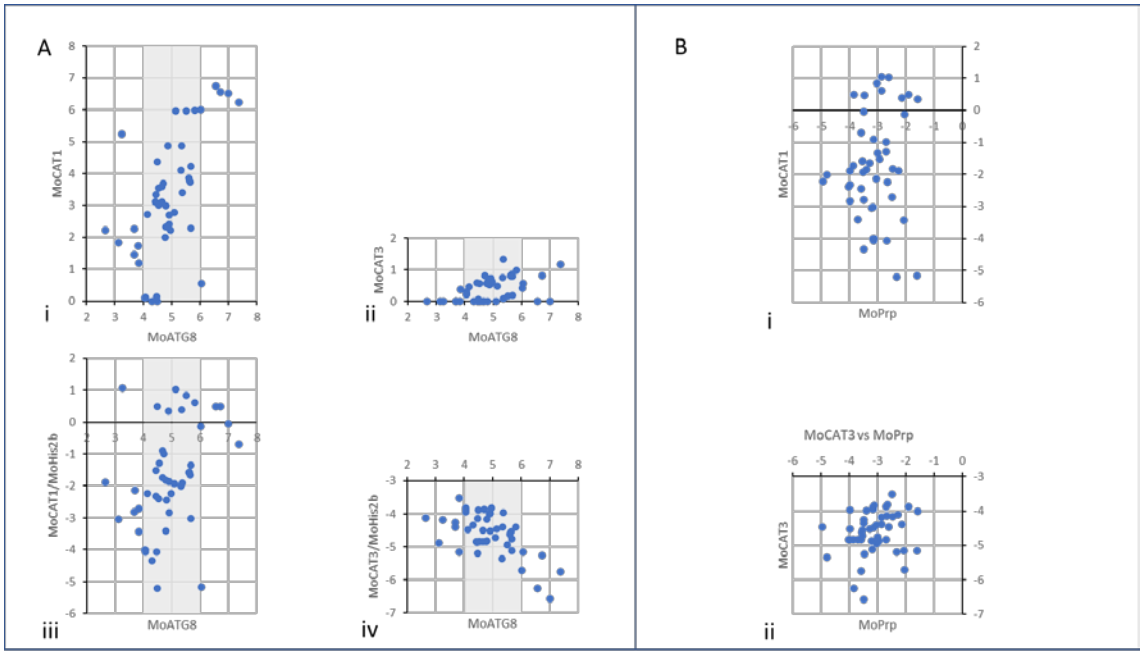

Figure 6. Catalase orthologues in versus MoATG8 (HPI). (**Ai-ii**) Expression of MoCAT1 and MoCAT3 versus MoATG8. (**Aiii-iv**) Expression of MoCAT1 and MoCAT3 versus MoATG8 normalized for growth rate (MoHis2B). All plots (A and B) are shown with equal scaling on both axes to facilitate comparisons. Grey areas (Ai-iv) mark the judged biotrophy/necrotrophy transition (Log2FgATG8=4-6). (Bi). Expression MoCAT1 and (Bii) MoCAT3 vs MoPrp. Both plots are growth-normalized and shown with equal scaling on both axes to facilitate comparisons. RMA regression gave P higher than 0.05 for no correlation with MoPrp. MoCAT1=0.59 and MoCAT3=0.14, indicating correlation not likely.

However, none of the two catalase orthologues is strongly positively correlated with the MoPrp gene (**Fig. 6.B**). That would be expected if any of these catalases helped the fungus defend against ROS-mediated DNA damages. It appears like MoPrp is not activated, although MoCat1 is activated, indicating that MoPrp might not be upregulated due to oxidative DNA damages. This non-regulation of MoPrp is a bit strange since the fungus needs to use catalase to withstand other damages when exposed to ROSs during necrotrophy (**Fig. 6A-i and ii**). Not activating or inhibiting the PARP gene can lead to increased mutations due to DNA damages [14,25].

F. graminearum responses to intrinsic NO or plant generated NO oxidative stress

F. graminearum has two nitric oxide dioxygenase genes FgNOD1 and FgNOD2 (**Table 2**). Both genes are sharply upregulated in the transition between biotrophy to necrotrophy (**Fig. 7-Ai,ii**), most likely because of plant generated NO instead of intrinsically generated NO that seem to dominate at low expression levels of ATG8 (HPI). This pattern is even more pronounced when normalizing for growth (**Fig. 7Aiii,iv**). The likely response to plant-generated NO in the transition between biotrophy to necrotrophy is made even more probable if, instead, the NOD expression is normalized for the main protein involved in intrinsic fungal NO formation. Now it can be seen that there seems to be a balance between FgNODs and FgCYP(NO,ERG) before the biotrophy-necrotrophy transition (**Fig, 7B**). Thus, plant NO stress most likely dominates inside the plant,

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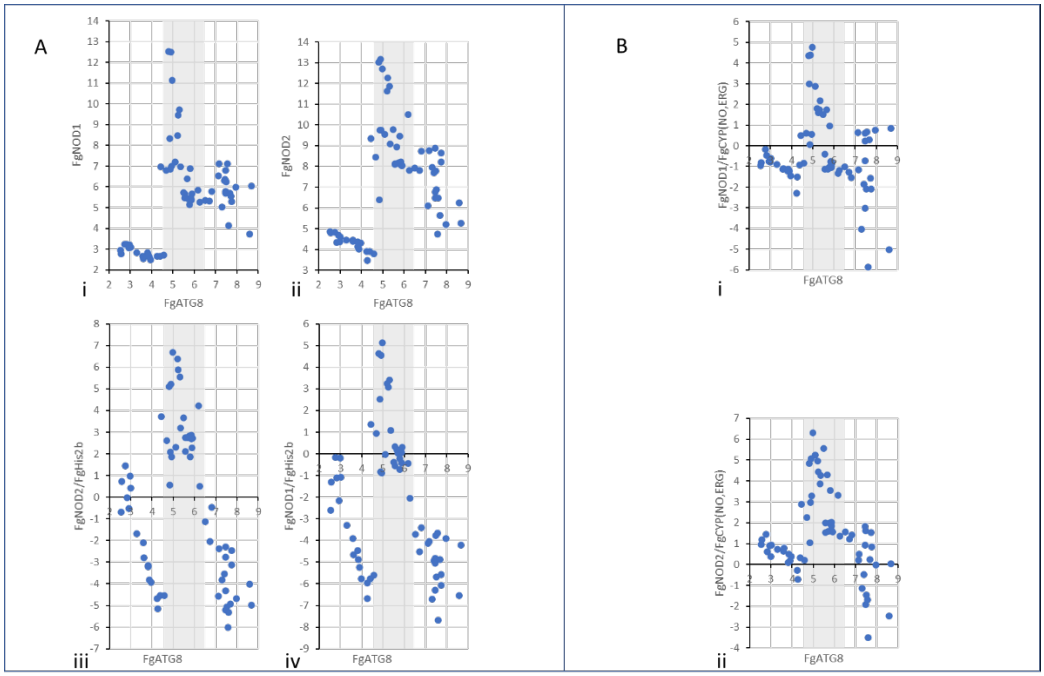

Figure 7. FgNOD1 and FgNOD2 in *F graminearum* vs. HPI (FgATG8). A and B without growth normalization C and D with growth normalization using FgHis2b. E and F with normalization for the NO forming CYP using FgCYP(NO,ERG). All plots (**A,B**) are shown with equal scale tick marks on both axes to facilitate comparisons. Gray areas mark the judged biotrophy/necrotrophy transition (Log2FgATG8=4.5-6.5).

while intrinsically produced NO probably dominates at low ATG8 levels before infecting the fungus and biotrophy starts. The ratio of the expression of the two NOD genes per FgCYP(NO,ERG) (FgNOD1 or 2/FgCYP(NO,ERG)) as an indicator for intrinsically produced NO versus FgATG8 was plotted to see if intrinsically dominated NO is more likely at low FgATG8 levels (HPI), and it is (**Fig. 8A**). It was also plotted against intrinsic NO generation indicated by FgCYP(NO,ERG) expression (**Fig. 8B**). The results indicate that after the biotrophy-necrotrophy transition intrinsically produced, NO is negatively correlated with NO defences suggesting that these NO defences are most likely against plant-generated NO.

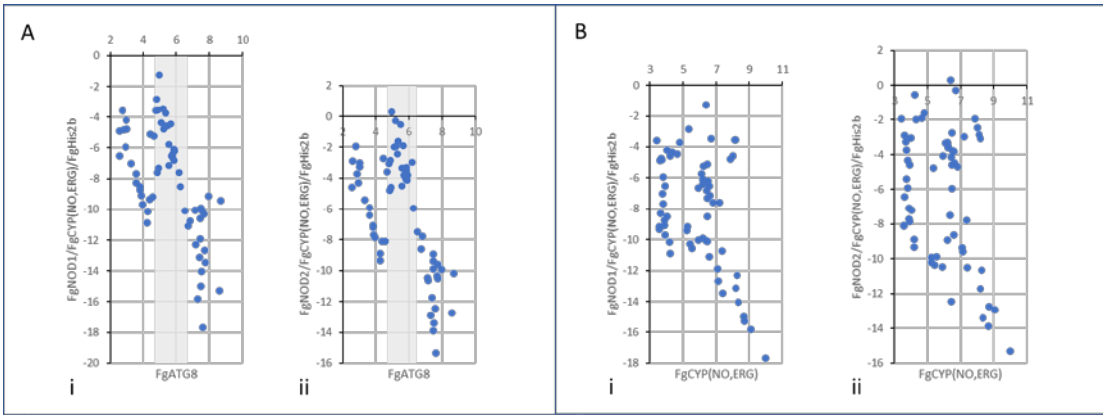

Figure 8. **A** shows NO stress (extrinsic) indicated as FgNODs/FgCYP(NO,ERG) is high at low HP and in the transition between biotrophy to necrotrophy but at high FgCYP(NO,ERG) expression (above 8-9) does not seem to be counteracted by NOD. **B** shows the same ratios plotted against FgCYP(NO,ERG) and shows that there is, in principle, an inverse relationship between intrinsic NO formation and defence against NO. Both plots (**A,B**) are shown with equal scale tick marks on both axes to facilitate comparisons. Grey areas (A) mark the judged biotrophy/necrotrophy transition (Log2FgATG8=4.5-6.5).

NO is highly mutagenic, and the expression of both NODs that indicate needed ROS defences due to NO is strongly correlated with PARP expression, suggesting that more DNA repair is needed at the high NO levels likely caused by the plant defences (**Fig. 9A-B**).

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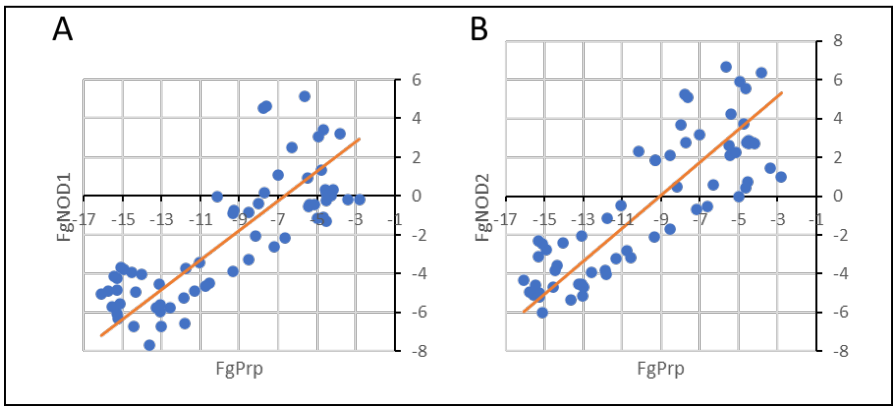

Figure 9A-B. The two FgNODs plotted versus FgPrp. Both are normalized for growth. An expected strong correlation was found, indicating that both NOds help the fungus against NO produced by itself and/or by the plant. Both plots are shown with equal scaling on both axes to facilitate comparisons. RMA regressions are shown as red lines. P for no correlated where FgNOD1=1.56E-15 and FgNOD2= 2.23E-18. The two correlations' slopes were not significantly different since, in pairwise comparisons, they showed overlapping 95% confidence intervals for their calculated slopes.

In *M. oryzae,* there is only one good BLAST hit for a NOD orthologue, MoNOD (**Table 2**). For *M. oryzae,* the regulatory pattern is less clear. However, as for *F. graminearum,* MoNOD is sharply upregulated in the transition between biotrophy to necrotrophy (**Fig. 10Ai**). In this case, this is probably also because of plant-generated NO instead of intrinsically generated NO but might also be aided by intrinsically formed NO in the necrotrophic stage (at high MoATG8 levels). This pattern is even more pronounced when normalizing for growth (**Fig. 10 Aii**) where it can be seen that the MoNOD is sharply upregulated in the biotrophy-necrotrophy transition.

MoNOD is highly expressed (**Fig, 10Aii**) at the same time MoNOD/MoCYP(NO,ERG) ratio is low (**Fig. 10Aiii**) when growth adjusted, indicating that NO likely comes from the plant. At the late stages of infection, it is also clear that NO from the plant probably plays a larger role since this expression ratio increases (**Fig. 10Aiii**).

The idea that plant NO stress likely dominates inside the plant while fungal intrinsically produced NO probably dominates at low MoATG8 levels (low HPIs before entering biotrophy) was tested further. The NOD gene expression ratio to MoCYP(NO,ERG) as an indicator for intrinsically produced NO versus MoATG8 was plotted to see if intrinsically dominated NO is more likely at low ATG8 levels and it is (**Fig. 10Aiv**). To further confirm that this ratio is negatively correlated at high levels, it was also plotted against MoCYP(NO,ERG) (**Fig. 10Bi**). During plant infection, gene expression of the gene for intrinsically produced NO seems to be negatively correlated with the gene necessary for NO defences (Fig. 10Aiv, Bi), similar to the case for *F. graminearum* (**Fig. 8B**).

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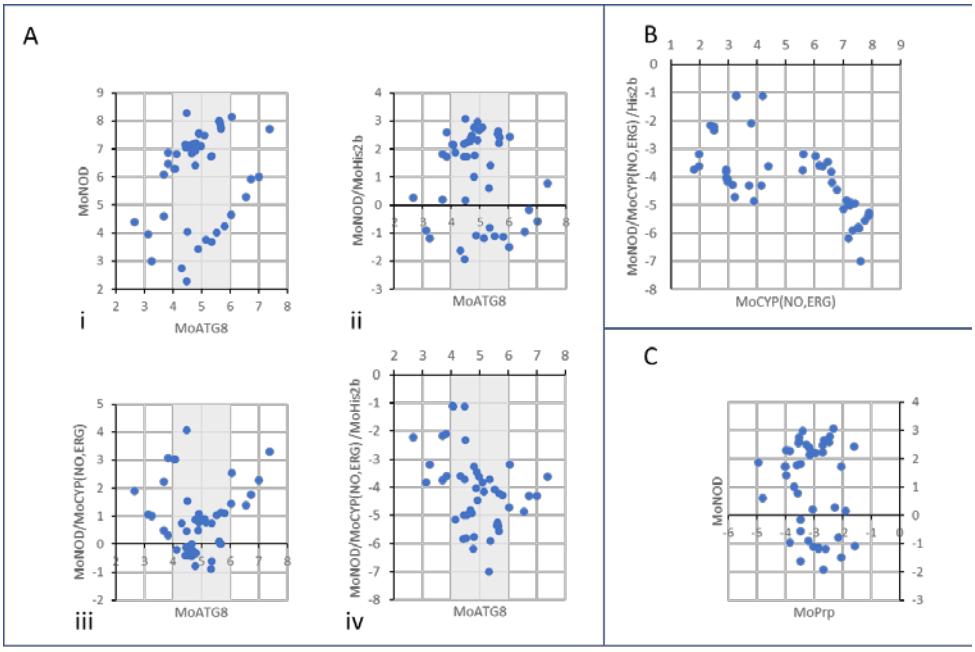

Figure 10. (**Ai**) MoNOD versus MoATG8 (HPI). (**Aii**) MoNOD growth-normalized as MoNOD/MoHis2b versus MoATG8 (HPI). (**Aiii**) MoNOD MoCYP(NO,ERG) normalized versus MoATG8 (HPI). (**Aiv**) The ratio in **Aii** growth-normalized versus MoATG8 (HPI). (**B**) Same Aiv but versus MoCYP(NO,ERG) showing an inverse relationship. (**C**) No Log2 relationship can be seen between MoNOD and MoPrp expression as would be expected if both were active to protect against NO as radical. All plots (**A-C**) are shown with equal scaling on both axes to facilitate comparisons. Grey areas (**Ai-iv**) mark the judged biotrophy/necrotrophy transition (Log2FgATG8=4-6)

Finally, since NO might damage DNA need to be repaired by MoPrp, MoNOD expression was compared to MoPrp expression. If MoPrp is active in repairing DNA damages caused by NO, there should be a positive correlation between MoPrp gene regulation and NOD gene regulation, as was noted for the similar relation in *F. graminearum* (**Fig. 9A-B**), but no such pattern is visible (**Fig. 10C**). This lack of regulation supports that in *M. oryzae,* increased DNA repair by MoPrp is not activated by the plant produced ROS as H_2O_2 and NO even if the fungus is stressed by these plant produced ROS.

Since this difference between FgPrp and MoPrp transcriptional activation was found, it was investigated if there can be differences in the "parylation toolbox" for the two fungi. Part of the PARP signalling pathway is the enzyme poly(ADP-ribosyl) glycohydrolase (PARG), the de-PARylation counterpart to PARP. PARG enzymes have been described in *Fusarium oxysporum* (FoPARG) [26], and we found an orthologue in *F. graminearum* (Table 2). The *F. graminearum* protein FgPrg has a high similarity and is identical to the FoPARG around the active site. Many orthologues PARGs can be found in fungi, and PARGs appear to be well conserved. However, there were no PARG orthologues to be found in *Magnaporthe* sp. To test if the PARG orthologue seems to be active in *F. graminearum,* FgPrg expression, and the FgPrp expression in the *in planta* data, was plotted together to see if they correlate and they are expressed in a 1/1 ratio at all stages of infection (**Fig. 11A-B**). For *M. oryzae*, the lack of a strong correlation of MoPrg with the ROS indicating genes (catalase and

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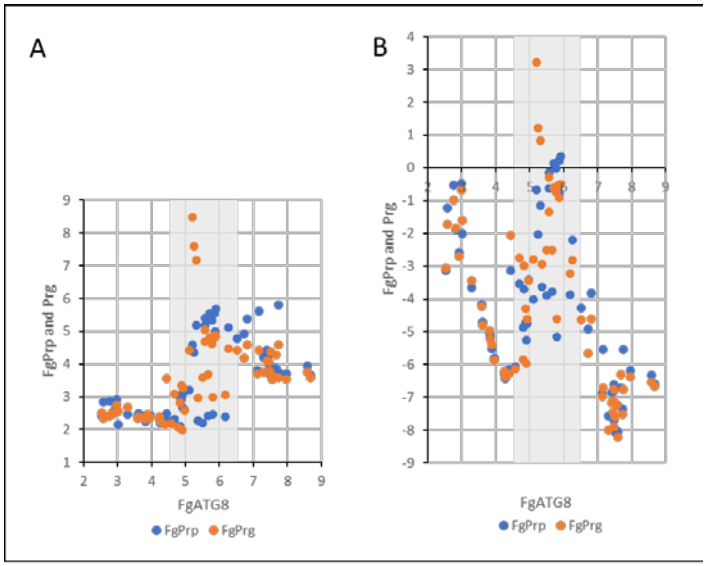

Figure 11 FgPrp putatively responsible for parylation, and FgPrg putatively responsible for de-parylation versus FgATG8 (HPI). A. The two genes expression versus FgATG8 (HPI). B. Growth-normalized. Blue dots=FgPrp and orange dots=FgPrg. Both plots (**A,B**) are shown with equal scale tick marks on both axes to facilitate comparisons. Grey areas mark the judged biotrophy/necrotrophy transition (Log2FgATG8=4.5-6.5).

NOD) (**Fig. 6B and 10C**) the ROS indicating genes (catalase and NOD) (**Fig. 6B and 10C**) and the lack of a MoPrg point to that MoPrp is not active, or at least not in the same way in *M. oryzae* as in *F. graminearum* or do not have the same role in repairing DNA-damage as the PARP/PARG system has in *F. oxysporum* [26].

Non-growing *F graminearum* challenged 0,1,2,4 h with bacterial MAMPs triggering NO formation.

Since transcriptome data for 113 transcriptomes of non-growing *F. graminearum* mycelia short-term exposed to bacterial MAMPs in water under non-growing conditions are available from our previous studies [17,22], it was tested if the maintenance specific network (**Fig1B**) is activated under these conditions. The ROS NO is produced in response to the bacterial MAMPs, and the FgCYP mainly causes the NO production (NO,ERG) [17,22] that is upregulated under these short-term non-growing conditions. FgATG8 is not differentially regulated; instead, CYP(NO,ERG) expression can be used as an indicator of increasing NO stress due to MAMPs challenges[17].

As can be expected, the genes responsible for producing arginine needed to produce NO are positively regulated with FgCYP(NO,ERG), purine synthesis FgCPA1 FgPrp FgPrg needed for DNA repair (**Fig. 12**). This regulation supports the notion that intrinsic NO production causes single-nucleotide mutations that need repair. Although the PARG gene FgPrg is upregulated, it does not entirely mirror the FgPrp gene found in the *in plant*a data (**Fig. 11**). This lack of mirroring can be due to the short time nature of the experiments (1-4h) since protein parylation by PARP is a speedy process [27] and quite fast again removed by PARG [28]. It could also indicate an increase in protein PARylation signalling that is a likely part of a fast-reacting fungal innate immune response [29].

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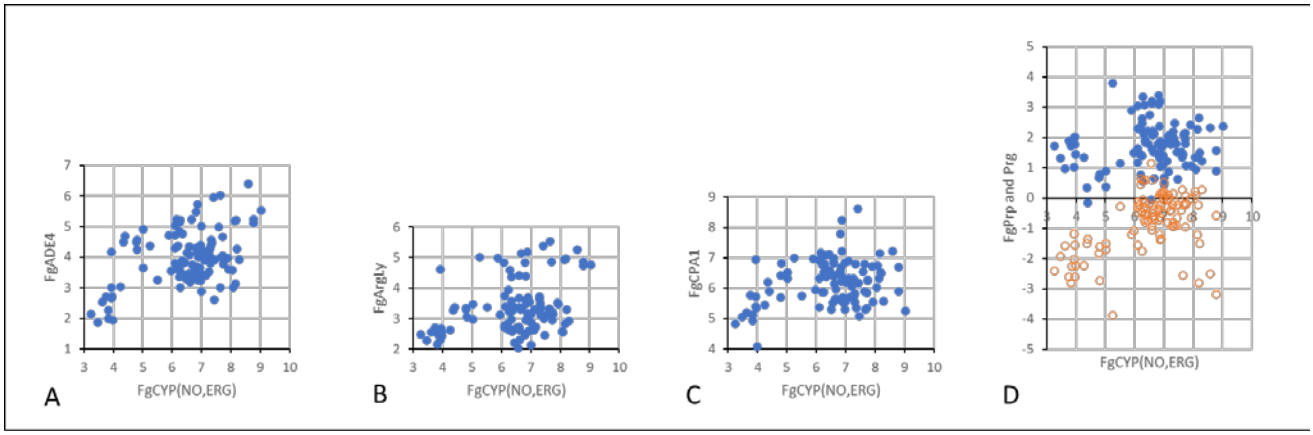

Figure 12 (**A-D**). Expression of 6 genes important for producing and counteracting NO when exposed to bacterial MAMPs. Five of the genes were plotted versus FgCYP(NO,ERG), known to form NO when exposed to bacterial MAMPs. (**D**) shows a double plot of the parylation gene FgPRG (blue dots) and the de-parylation gene FgPrg (orange dots). All plots are shown with equal scaling on both axes to facilitate comparisons.

ROS counteraction seems to be mainly detected by the increased oxidative state through the transcriptional regulator sensor dependent on oxidative stress formed S bridges in the stress transcription factor YAP1 in yeast and its *F. graminearum* orthologue FgAP1 [30]. Thus, the upregulation of the five catalases are most likely indicative of general oxidative stress rather than specific sensing of NO or other intrinsically generated ROSs (**Fig. 13**)

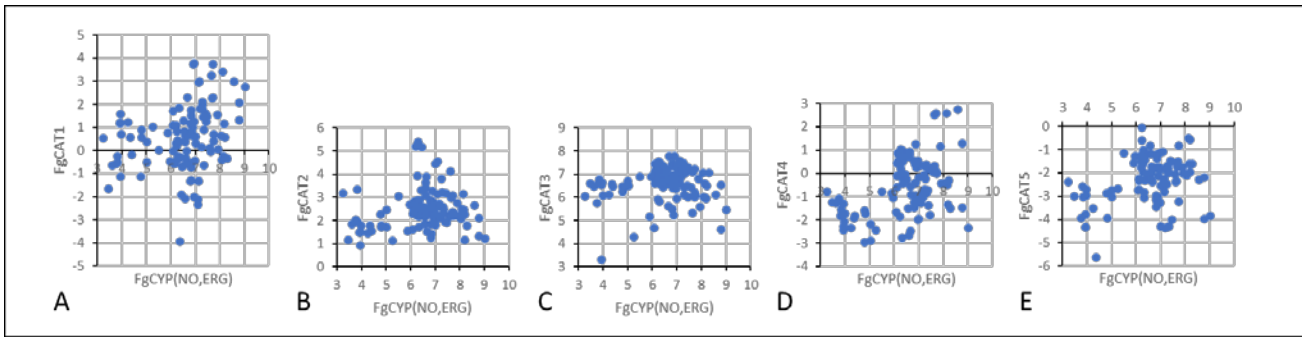

Figure 13. (**A-E)** Catalase gene expression seems to be higher at higher NO gene expression. All plots are shown with equal scaling on both axes to facilitate comparisons.

The two NODs necessary to regulate NO concentrations specifically are upregulated with increased expression of the NO generating FgCYP(NO,ERG), especially at the higher levels (**Fig. 14Ai,ii**). NOD1 is localized in the cytoplasm and the nucleus, while NOD2 is not in the nucleus [17]. The FgNOD1/CYP(NO,ERG) ratio can be expected to be negatively correlated with CYP(NO,ERG) to reach high levels of bacterial detection signalling when exposed to MAMPs. Thus it is expected that a negative correlation between FgNOD1/CYP(NO,ERG) ratio and Fg(CYP(NO,ERG) will be found, and that is indeed the case (**Fig. 14Bi,ii**). There is also a negative correlation between FgNOD2/CYP(NO,ERG), but it seems less tightly co-regulated. FgNOD2 is located in the cytoplasm and cytoplasmic puncta [17]; it is less likely directly involved in affecting NO-induced transcription factors activity. Since FgNOD1 is located in the nucleus and cytoplasm [17] and probably the signalling system's central part, lack of NOD1 activity allows NO to affect transcription factor activation downstream [17]. In support of this, the expression of FgNOD1 versus Fg(CYP(NO,ERG) is not strongly responding but roughly constant (+-Log2=1) over most of the Fg(CYP(NO,ERG) expression range (**Fig.14i**).

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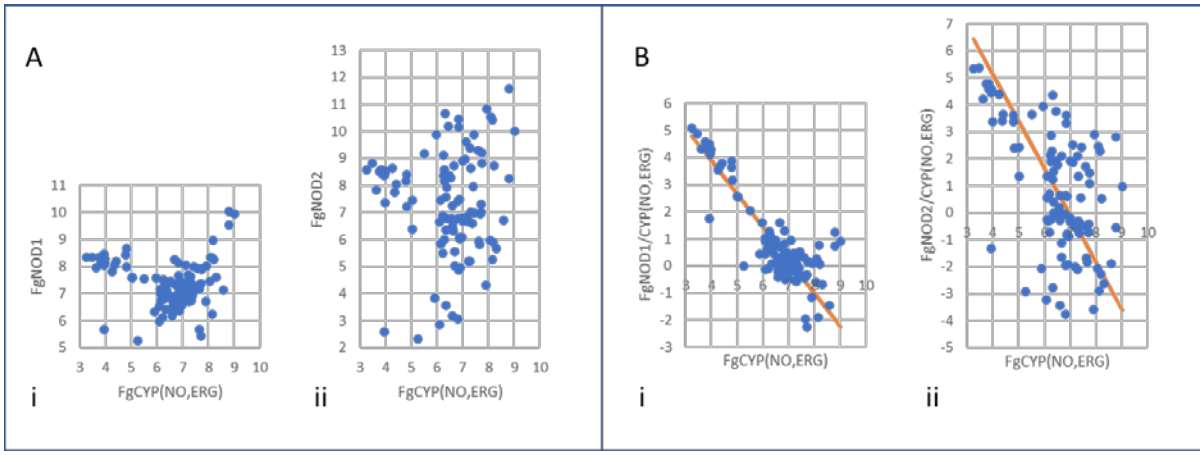

Figure 14. (**Ai-ii**) Both FgNOD genes increase in activity with NO gene expression, especially at the higher levels over 8. (**Bi-ii**) FgNOD/FgCYP(NO,ERG) decreases with increasing FgCYP(NO,ERG), indicating increased NO signalling. FgNOD1 localizes in the whole cytoplasm, and the nucleus [17] is negatively correlated and probably the main counterpart to FgCYP(NO,ERG). RMA regression (**B**) is shown as red lines. P for not correlated where FgNOD1/CYP(NO,ERG)= 2.77E-31 and FgNOD2/CYP(NO,ERG)=2.16E-10. Both correlations' slopes were not different since, in pairwise comparisons, they showed overlapping 95% confidence intervals for their calculated slopes. All plots are shown with equal scaling on both axes to facilitate comparisons.

Finally, under these conditions, the higher the level of NODs, the lower the concentration of NO should be, and consequently, there should be fewer DNA damages needing repair by PARP. Thus, NODs and PARP genes are expected to be negatively correlated, and they are (**Fig. 15**).

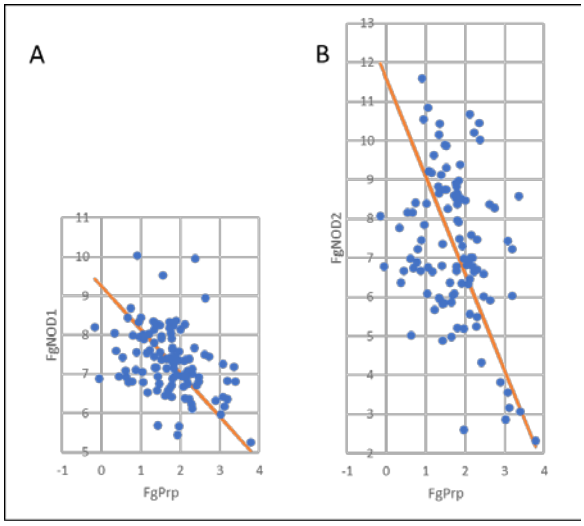

Figure 15. A-B the two FgNODs versus FgPrp. Negative correlations imply that when the FgNODs are high, there is less need for FgPrp under these non-growing conditions when bacterial MAMPs challenge the fungus since the FgNODs keep NO concentration low. RMA regression is shown as red lines. P for not correlated where FgNOD1= 2.35E-04 and FgNOD2= 3.43E-04. Both correlations' slopes were not different since, in pairwise comparisons, they showed overlapping 95% confidence intervals for their calculated slopes.

Potential relative roles of autophagy and apoptosis during the infection stages (HPI) in both fungi

In *Metarhizium robertsii*, belonging to the same order as *F. graminearum* (Hypocreales), the expression of MrBI-1 has been identified as linked to apoptosis [31]. Apoptosis is needed to empty hyphae (autolysis) for the use of resources in other hyphae (reallocation of resources) or production of conidia [13]. There is only one orthologue to this gene in *F. graminearum*, FgBI1 (**Table 2.**). When investigating *F. graminearum* growth measured as FgHis2b expression versus HPI as measured by FgATG8 expression (**Fig. 16Ai**), FgHis2b expression increases different rates during the course of infection. However, FgATG8/FgHis2b expression that is indicative of the use of internal stored resources is high early, then drops during biotrophy, increases in the transition to necrotrophy, and then drops going into necrotrophy for increasing during the last conidiation stages of

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necrotrophy (**16Aii**). The mirror image of this, FgHis2b/FgATG8 (**16Aiii**), is consequently indicative of the use of external resources, in this case, plant biomass.

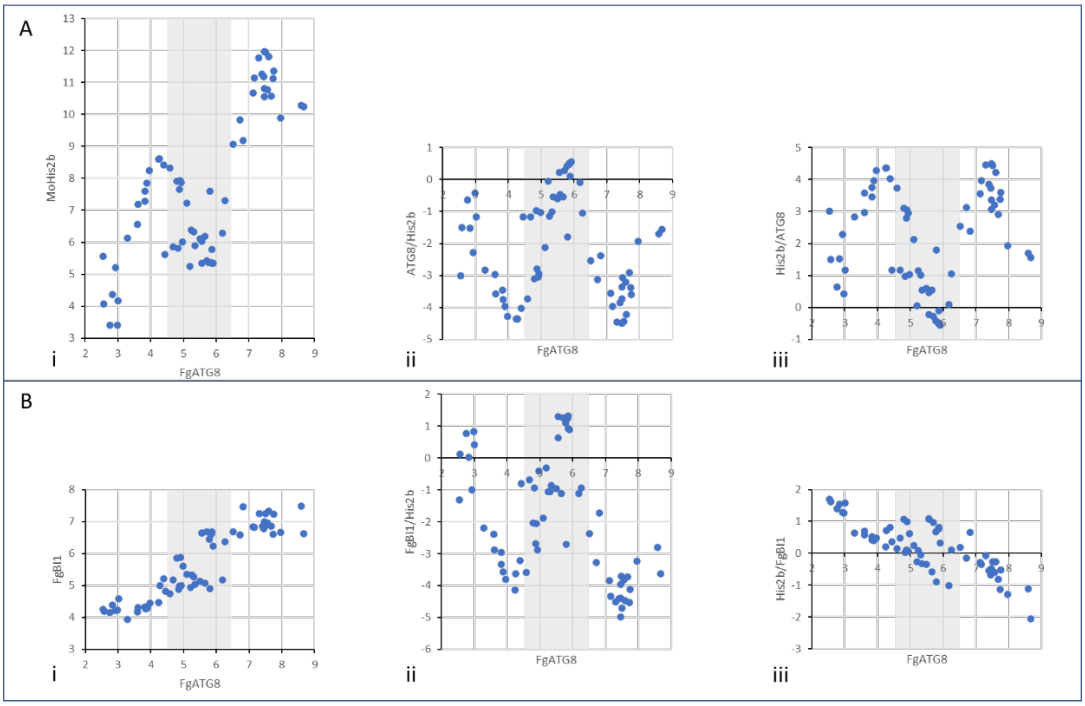

Figure 16. Relationship between His2b (DNA synthesis and growth) and FgATG8 (use of stored resources and repair) versus FgATG8 (HPI). (**Ai**) With increasing HPI (FgATG8), the fungus increases in growth (FgHis2b). (**Aii**) Growth corrected FgATG8 expression versus FgATG8 to indicate when internal resources are used. (**Aiii**) The mirror image of B shows when plant resources are used. (**Bi**) Showing that the apoptosis indicated by FgBI1 increases with FgATG8 (HPI). (**Bii**) Growth corrected FgBI1 indicates apoptosis is highest before plant entry and in the biotrophy necrotrophy transitions. (**Biii**) Compared to autophagy, apoptosis decrease with FgATG8 (HPI). All plots are shown with equal scaling on both axes to facilitate comparisons. Grey areas mark the judged biotrophy/necrotrophy transition (Log2FgATG8=4.5-6.5).

The expression of FgBI1 increases with FgATG8 (HPI) as expected (**Fig. 16Bi**) since the reallocation of resources from non-productive hyphae to productive hyphae are keys in mycelium development [32,33]. Consequently, growth-normalized FgBI1 expression vs HPI (ATG8) also looks like the ATG8 curve (**Fig. 16Aii and Bii**). Apoptosis seems to play a slightly decreasing role compared to autophagy with increasing HPI (ATG8) since the ratio FgBI1/FgATG8 decrease with HPI (ATG8) (**Fig. 16iii**).

Figure 16 and previous figures show that five stages for wheat infection by *F. graminearum* can be suggested (**Table 3**).

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Table 3. Stages for the infection of rice by *F. graminearum* identified as identified by the regulation

Stage No.	Activity	Stage	Log2 FgATG8 expression
1	Conidia start growing mainly on stored resources	Arrival-pre-penetration stage	<3
2	Infection hyphae enter the plant and grow considerably without stress from plant defences	Biotrophic stage	3-4.5
3	Plant discovers the invading and mounts defences. Biotrophic growth resources might also suddenly go scarce, causing intrinsic ROS-stresses.	Biotrophy-necrotrophy transition stage	4.5-6.5
4	Plant cells are killed or inhibited by DON. Growth resumes and reaches a higher growth rate.	The necrotrophic stage	6.5-8
5	Plant cells are consumed, and new growth stops, and fungus uses autophagy and a fair bit of apoptosis to evacuate vegetative mycelium and form spores.	The "emigration"-conidiation stage	>8

The situation for *M. oryzae* is similar but also different. The growth rate measured as His2b expression does not increase substantially until the transition to necrotrophy and the necrotrophic stage (**Fig. 17A**). At the switch to necrotrophy and in the necrotrophic stage, autophagy's relative importance to growth is higher, indicating stress and/or reallocation of nutrients through autophagy (**Fig. 17A-C**). On the other hand, apoptotic emptying of hyphae (autolysis) seems to be much more important at an earlier stage in *M. oryzae* than in *F. graminearum.* The emptying of vegetative hyphae can be indicative of considerable simultaneous both growth and sporulation triggered at the transition from biotrophy to necrotrophy as also apoptosis MoBI1 expression increases and stays high at the same level of MoATG8 expression (HPI) growth-normalized (**Fig. 17Bii**) and ATG8 normalized (**Fig. 17Biii**). However, for this fungus, there also seems to be a slight decrease before a final spurt in apoptosis (aiding conidiation) at the very high levels of MoATG8 expression (HPI) (**Fig. 17Bii-iii**).

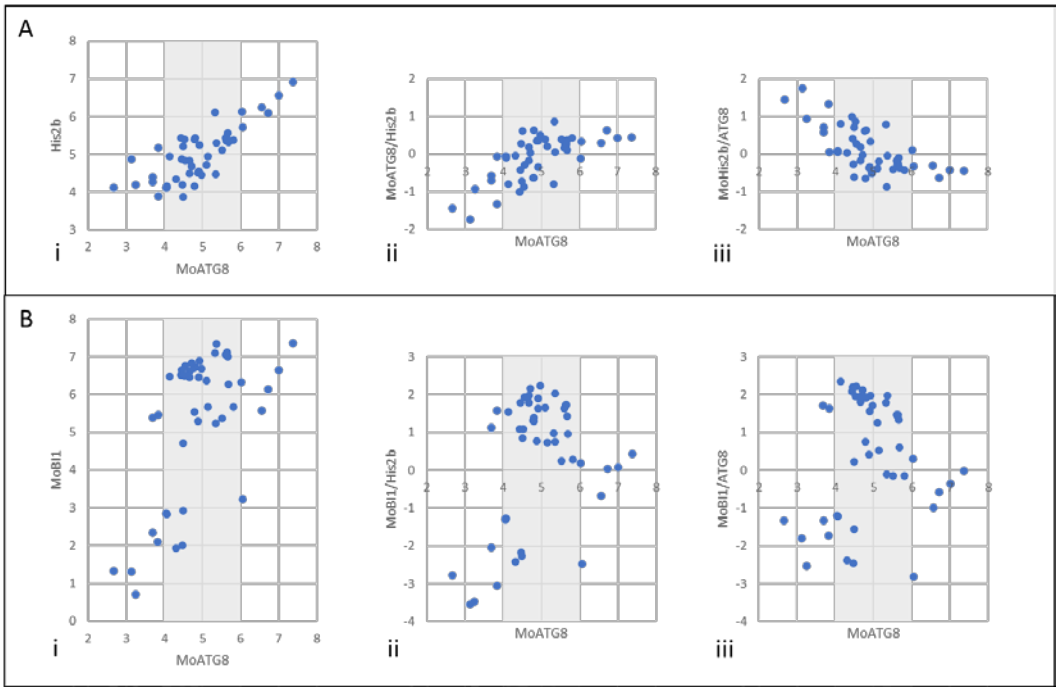

Figure 17 Relationship between His2b (DNA synthesis and growth) and MoATG8 (use of stored resources and repair) versus MoATG8 (HPI). (**Ai**) With increasing HPI (MoATG8), the fungus increases in growth (MoHis2b). (**Aii**) Growth corrected MoATG8 expression versus MoATG8 to indicate when internal resources are used. (**Aiii**) The mirror image of B shows when plant resources are used. (**Bi**) Showing that the apoptosis indicated by MoBI1 increases with MoATG8 (HPI). (**Bii**) Growth corrected MoBI1 indicates that apoptosis is highest in the Biotrophy necrotrophy transitions. (**Biii**) Compared to autophagy, apoptosis is high at intermediate MoATG8 expression (HPI).

All plots are shown with equal scaling on both axes to facilitate comparisons. Grey areas (**A,B**) mark the judged biotrophy/necrotrophy transition (Log2FgATG8=4-6)

Figure 19 indicates that four stages of rice infection by *M. oryzae* can be suggested (**Table 4)**.

Table 4. Stages for the infection of rice by *M. oryzae* identified as identified by the regulation

Stage No.	Activity	Stage	Log2 FgATG8 expression
1	No detectable extra stress in the pre-penetration stage or this stage is short, so not identifiable in the transcriptomic data		
2	The fungus infects and starts using plant resources biotrophically for slow growth/maintenance	Biotrophic stage	<4
3	Plant discovers the invading and mounts defences. Biotrophic growth resources might also suddenly go scarce, causing intrinsic ROS-stresses. The plant cells are killed, and the nutrients from the plant cells' degradation speed up fungal growth and increase stresses imposed by the plant defences. The plant ROS stresses induce melanization of fungal cell walls [34]. The fungus starts emptying mycelium to form conidia.	Biotrophy-necrotrophy transition stage	4-6
4	Plant cells are killed, and resources become scarce autophagy and apoptosis are used to empty mycelium and fill spores. This spore filling is probably caused mainly by apoptosis that starts already early in the transition from biotrophy to necrotrophy	The necrotrophic stage	>6

Compared to *F. graminearum,* the analysis indicates that *M. oryzae* does not have an extended necrotrophic stage with a buildup of vegetative mycelium but uses the host biomass and starts forming conidia and emptying the mycelium already at the start of the necrotrophic stage. It also appears that apoptosis could play a more critical role in this process for *M. oryzae* than for *F. graminearum*. This difference could be why relatively small lesions are seen for *M. oryzae* on rice leaves [6] compared to whole wheat grains and whole wheat heads or seedlings infected by *F. graminearum* [8]. The available resources for the Functional Mycelium Unit (FMU) [32,33] that is the fungal individual seems to be much smaller in *M. oryzae* and is reflected in short to non-existent “happy” non-stressed growth period in the necrotrophic stage.

Conclusion

Testing the hypothesis: The analysis supports the hypothesis presented in the introduction and discussed in the Results and Discussion (above). To summarize this, a conceptual model is presented (**Fig. 18A)**. In this Figure 18, it can be seen that the genes responding to stresses and that are needed for maintenance are regulated in a W fashion with HPI (LOG2 ATG8 expression) in F. graminearum (**Fig. 18B and C**). For *M. oryzae,* the stress is similar in the transition between biotrophy to necrotrophy, but the stages before and after are generally not characterized by increased stress except for catalases indicating plant defences induced during necrotrophy (**Fig. 18A**). The shape of the response profile is not a W but more like a “wizard hat” (**Fig. 18B and C).**

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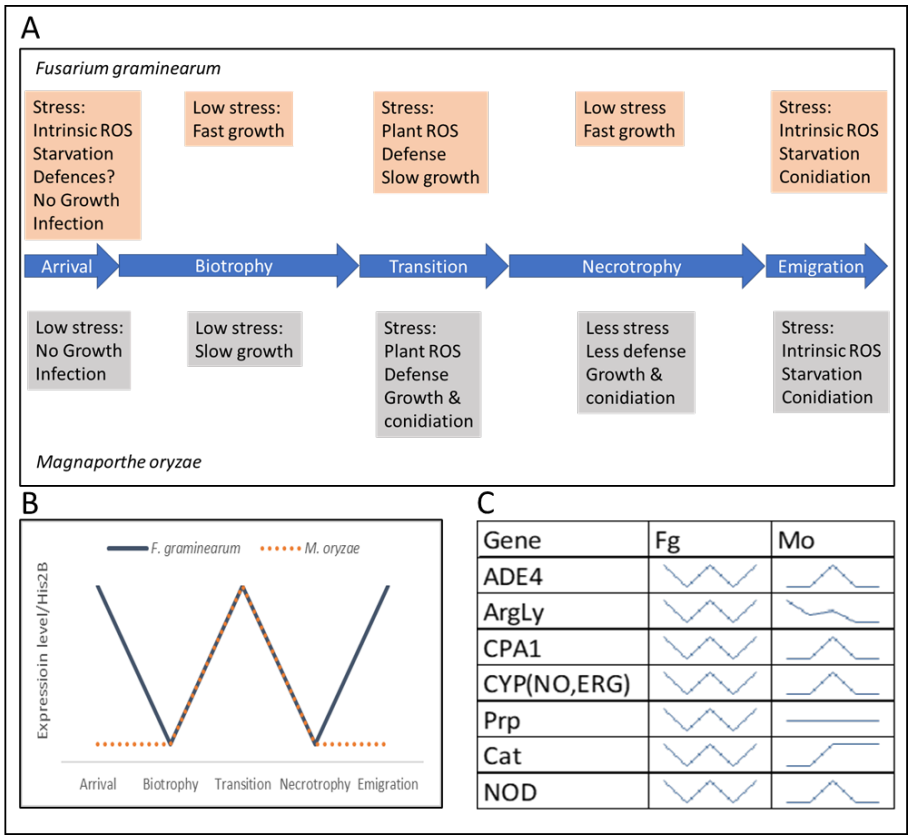

Figure 18. A conceptual model for the plant interaction progression concerning the genes monitored from "arrival" to "departure" for the two plant pathogens, both having a biotrophic stage and a necrotrophic stage, and a transition stage in between. The model highlights the found similarities and differences. (**A**) "Timeline" of the different stages. (**B**) The generalized growth-normalized gene expression profile for the genes in Figure 1 showing the W-responses for *F. graminearum* and the "wizard hat" responses for *M. oryzae*. (**C**) Comparing the generalized gene-expression profiles in *F. graminearum* and orthologues in *M. oryzae* for the genes in Figure 1. See Figure 1 legend for gene abbreviations.

Differences between *F. graminearum* and *M. oryzae* in PARP/PARG gene activation, and possible consequences for their respective co-evolution with their hosts: The only one of the genes identified to be important in the network (**Fig. 1 and 2**) that did not follow the expected general pattern with increases in expression in the biotrophy/necrotrophy transition for both fungi was PARP needed for DNA repair of point mutations [25]. The PARP gene FgPrp is activated *in planta* for *F. graminearum,* but that does not happen for the orthologue MoPrp in *M. oryzae* (**Figs. 2E, 2J, 3E, 3J, 5A-E, 6B, 9, 10C**). Besides, *M. oryzae* lacks an orthologue to the FgPrg necessary for de-PARylation. *M. oryzae* is mainly clonal [9], while *F. graminearum* is commonly sexually reproducing [8]. A non-functioning or non-reacting PARP/PARG system should, during infection and exposure to plant ROS defences, lead to increased mutation rate as have been shown for an *F. oxysporum* PARG mutant [26]. *F. graminearum* and *M. oryzae* are relatively closely related. However, they differ in generating offspring genetic variation. *F. graminearum* uses sexual reproduction. The fungus is also homothallic and self-fertile, having both necessary mating-type loci making it possible to combine favourable mutations in haploid nuclei within the same thallus [7,8,35]. As mainly a clonal fungus [9], *M. oryzae* is dependent on mutations within the same nucleus and/or epigenetic changes to overcome new host resistance development. In conclusion, host ROS caused multiple mutations due to DNA-damages is mainly negative and needs to be corrected in *F. graminearum.* However, for *M. oryzae,* although immediately lowering infection success for the fungus, multiple DNA damages will speed up mutation rate [26] and shorten the number of generations necessary to break plant resistance. Thus, in a pathogen with frequent sexual recombination, especially if this is necessary for infection or triggered during infection, the sexual recombination can generate collections of beneficial mutations in offspring strains to overcome plant resistance development. If PARP/PARG is not activated or lost in a clonal pathogen, the host defences will generate stress that will increase

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the pathogen's mutation rate. Both and especially the last creates a Red Queen dynamics for the host-pathogen relationship [36] since increased resistance in hosts creates increased stress in the pathogen that directly creates increased pathogen variation. This results in the fungus being able to break host resistance creating more disease in the host. More resistant hosts survives better when the pathogen is present, and increased resistance in host……..and so on and so on (**Fig. 19**). It could be expected that such an automatic host resistance caused increased mutation rate as indicated for *M. oryzae* is more beneficial to the fungus if fungal host range is narrow and infection cycles are short with ample production of infectious conidia.

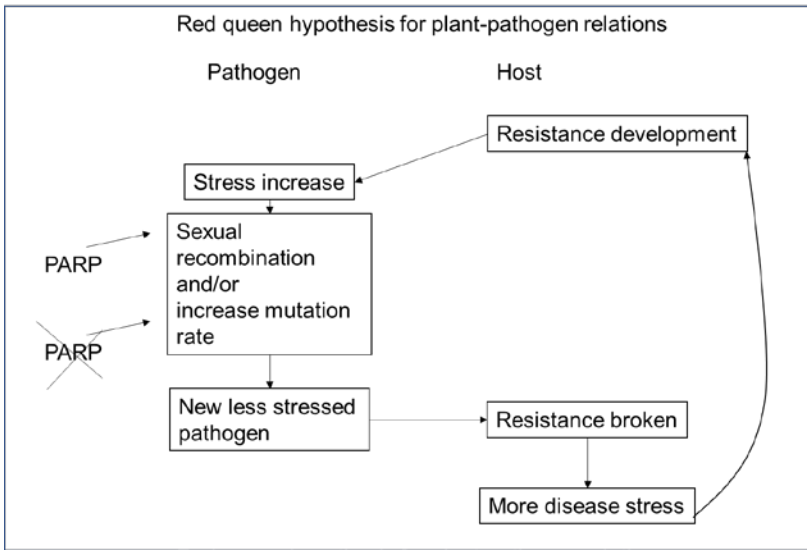

Figure 19. Stress caused by the host causes sexual recombination or increased mutation rate of the fungal pathogens. A non-functioning PARP (PARP/PARG) can potentially increase the mutation rate to benefit a clonally reproducing pathogen.

Eco-physiological ranges of abiotic and biotic conditions and gene expressions – the advantage of inferring gene functions from large sets of transcriptomic data: Growth media are generally far too rich and outside eco-physiological ranges for the microorganism of study [33,37]. Under such conditions, genes are likely to show gene regulation mainly due to these artificial conditions and additional gene functions not seen within normal ranges [38]. Complete deletion of a gene not only removes that gene function from an early developmental stage (for example, spore germination) and can sometimes be considered lethal even if its role is not crucial at later stages. For most genes expressed at different levels during growth in the natural environment, a complete deletion creates a physiological situation far outside what the organism can theoretically meet in nature and is prone to cause artefacts in transcriptional adaptation and genetic compensation [39]. Artificial overexpression can cause similar problems by inducing expression levels far outside natural ranges. Protein overexpression is known to cause artefacts by forming protein complexes [40]. Such overexpression is especially a problem if the gene product is cytotoxic if produced in surplus, like histones [11]. Analyzing correlations between gene expression of genes belonging to physiologically relevant connected processes in transcriptome datasets from natural conditions (not standard lab media) like in the present study can potentially overcome some of these limitations and should be interrogated more frequently by researchers. This insight also calls for developing tools for conditionally up or downregulation of genes and graded regulations. It also calls for *in-vitro* studies under environmentally more relevant nutrient availabilities and composition than routinely used. Most problematic for understanding the ecological relevant roles for genes is perhaps that no fungi are growing in isolation in nature. They are always surrounded by their characteristic microflora of bacteria [41] and other microorganisms. Lack of these interactions is also why transcriptomic studies of pathogens during natural infection of plants can be better trusted for inferring gene functions than "normal lab media". For the organism in this study's focus, there is a lack of transcriptomic studies for *F. graminearum* on debris at different ages since incorporating the fungus into the soil on and in debris is part of its life cycle. For this fungus, there is also a lack of transcriptomic studies from the rhizosphere of seedlings at different times since a root tip passed since coming close to an inoculum might trigger fungal rhizosphere

 doi:10.20944/preprints202012.0773.v2

activities [42,43]. Such studies will be tricky since the amount of RNA will be tiny, but they are today possible [44]. For *M. oryzae,* it is evident in the downloaded transcriptomic data [10] that the amount of RNA has not been large since many genes lack expression data under many conditions. Thus, similar techniques for using a small amount of RNA might solve future research problems.

Author Contributions

Conceptualization, Stefan Olsson; Data curation, Stefan Olsson and Bjoern Oest Hansen; Formal analysis, Stefan Olsson; Funding acquisition, Zonghua Wang and Guodong Lu; Investigation, Stefan Olsson and Quingfang Li; Methodology, Stefan Olsson and Bjoern Oest Hansen; Resources, Stefan Olsson, Osakina Aron, Hongchen Li and Wenhui Zheng; Supervision, Stefan Olsson, Wei Tang and Zonghua Wang; Validation, Stefan Olsson; Visualization, Stefan Olsson; Writing – original draft, Stefan Olsson; Writing – review & editing, Stefan Olsson, Osakina Aron, Bjoern Oest Hansen, Zonghua Wang, Guodong Lu and Wenhui Zheng.

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