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**Nutritional factors modulating plant and fruit susceptibility to pathogens: BARD Workshop, Haifa, Israel, February 25-26, 2018**

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**Abstract**

The molecular dialog between fungal pathogens and their plant hosts is governed by signals from the plant, secreted pathogen effectors and enzymes, and the plant immune system. There is an increasing awareness that nutritional factors are also central to fungal-plant interactions. Nutritional factors include carbon and nitrogen metabolism, local pH and redox state, and manipulation of host metabolism by secreted pathogen effectors. A diverse combination of approaches from genetics, biochemistry and fungal and plant cell biology addresses these questions, and a workshop whose abstracts accompany this note was held in 2018 to bring these together. Questions were asked about how the lifestyles and nutritional strategies of eukaryotic filamentous phytopathogens are related to the metabolic architectures and pathogenic processes affecting both plant hosts and their pathogens. The aim for future work will be to provide metabolism-based strategies for pathogen control.

Nutritional factors are central to the molecular dialog between fungal pathogens and their plant hosts. Indeed, the distinction between a signal and a nutritional factor is often merely one of substrate concentration. Submicromolar levels may be enough for a ligand to activate a receptor, while millimolar or even higher concentrations can drive metabolic reprogramming. Carbon and nitrogen metabolism, local pH and redox state, and manipulation of host metabolism by secreted pathogen effectors are a few of the areas under study. As an example, consider the initial biotrophic phase of rice blast infection (Figure 1). *Magnaporthe oryzae* (synonym of *Pyricularia oryzae*), a leaf pathogen, grows for the first hours of infection in intimate contact with living rice cells. By 44 hours post inoculation (hpi), when invasive hypha (IH) are moving from the first infected cell into neighbouring cells, GFP labeled Bas4 (an apoplastic effector used to probe the integrity of the apoplastic compartment) surrounds invasive hyphae, while mCherry labeled PWL2 accumulates in the Biotrophic Interfacial Complex (BIC, Fig. 1A top panel). Treating infected cells with the autophagy inhibitor 3-methyl adenine at 36 hpi prevents cell-to-cell movement and, by 44 hpi, erodes the BIC and the biotrophic interface forming the apoplastic compartment (Fig. 1A, middle panel). Stimulating autophagy with amiodarone treatment increases cell-to-cell movement (Fig. 1A, middle panel). Thus, autophagy, and its control in response to the metabolic status of the fungal cell (Sun et al. 2019) is central to the mid-biotrophic growth stage. This metabolism-related phenotype is mirrored by the role of a conserved cell signaling pathway (Fig. 1B): invasive movement from the first infected cell to neighboring cells cannot occur in the absence of signaling through the Pmk1 MAP kinase pathway (Sakulkoo et al. 2018). The perceived nutrient environment that triggers autophagy during biotrophy is not well understood. It is clear, though, that early biotrophic growth requires the metabolism of glucose through the pentose phosphate pathway (Fernandez et al. 2014b). This depends on the function of the cell intrinsic glucose-6-phosphate/ NADPH sensor Tps1 (Wilson et al. 2010), while certain exogenous nitrogen sources such as some amino acids and adenine appear less readily available. The data suggest that nutrient-monitoring pathways (likely involving Tps1 and the autophagy-controlling Target of Rapamycin (TOR) signaling pathway) adapt *Magnaporthe* for growth in a glucose-rich, nitrogen-limiting environment (Fernandez et al. 2014c).

Furthermore, in fruit pathogens, infections remain quiescent until the fruit mature and ripens: the germinated spore or the germinated appressorium can only develop short primary hyphae in the unripe tissue, but its growth is activated and the fungus develops a necrotrophic colonization upon fruit ripening where higher sugar availability signals for the activation of colonization.

A workshop in spring 2018 on nutritional factors in the interaction of fungal plant pathogens with their hosts brought together fungal geneticists, plant pathologists and fungal cell and molecular biologists at the Technion, Israel Institute of Technology in Haifa (https://bardworkshop.net.technion.ac.il/). Timing together with the 14th European Conference on Fungal Genetics (ECFG14) led to a unique meeting in this Mediterranean port city, with the first day's venue on the slopes of Mount Carmel and the second day's near the sea. The focus of this meeting was on how the lifestyles and nutritional strategies of eukaryotic filamentous phytopathogens are related to the metabolic architectures and pathogenic processes of both host and pathogen. Oomycetes and fungi have evolved three general lifestyles to infect and feed on host crops: i. necrotrophs kill host cells before feeding off the destroyed tissue; ii. biotrophs grow and complete their lifecycle in living plant tissues; iii. hemibiotrophs undertake a period of symptomless biotrophy before switching to necrotrophy. The aim of the workshop was, from the many variations on these general patterns, to reach general principles regarding the role of fungal metabolism during host infection, and in doing so, to provide some new directions for the control of pathogens in agriculture. The following discussion is organized along the fungal life (and infection) cycle, but there is, obviously, overlap between the topics, as metabolic factors are involved in all aspects of fungal development.

## *1. Sporulation and germination*

The infection cycle of plant pathogens overlaps partially or entirely with the life cycle, depending on spore type, mode of dispersal, and whether the pathogen depends strictly on its host or grows alternatively as a saprophyte. Germination and sporulation can be thought of as two ends that are joined to make up the life cycle. Phytopathogenic fungi often have more than one spore type, perhaps representing a division of labor during the infection cycle. Several ascomycetous fungi generate dimorphic asexual conidia. This includes saprophytic species like

*Neurospora crassa*, but also plant pathogenic fungi such like *Colletotrichum graminicola* and *Magnaporthe oryzae* (Panaccione et al., 1989; Kato et al., 1994; Maheshwari, 1999). Typically, only one spore type is used for scientific investigations and therefore the role of the additional type often remains obscure. However, current studies underline a probable task sharing among the dimorphic conidia within infection and life cycles. In *N. crassa*, the typically investigated brightly orange macroconidia are described to serve the distribution of the fungus. The formation of these conidia is controlled by the circadian clock and the resulting spores are very efficient in early colony formation followed by the generation of female ascogonia (Feldman and Hoyle, 1973; Fleißner and Herzog, 2016). The microconidia, however, are less abundant and form independently of the clock. It is speculated that during clock-caused absence of macroconidia, microconidia can be spread readily, serving as spermatia to fulfill the sexual cycle in surrounding young *N. crassa* colonies (Maheshwari 1999). For phytopathogenic species, likewise, a division of labor between dimorphic conidia is plausible. In 2014, a specific distribution of macro- and microconidia in planta was described for the rice blast fungus *M. oryzae* (Zhang et al. 2014). Whereas the well-studied three-celled macroconidia are formed on necrotic lesions, the microconidia can be isolated from infected plant tissues by gentle grinding and serve the distribution of the disease via the vascular system. Since microconidia are able, albeit to much lower percentages, to cause rice blast symptoms, a role in the pathogenicity cycle of this important pathogen is likely (Zhang et al. 2014). The macro- and microconidia of the foliar leaf pathogen *C. graminicola* show distinct formation loci similar to *M. oryzae*: whereas the smaller oval are constricted from hyphae in liquid cultures and can be isolated from infected plant cells, the larger falcate conidia are born from short conidiophores in asexual fruiting bodies formed inside the lesion on infected plant (Panaccione et al., 1989; Sukno et al., 2008). Germination of falcate conidia is regulated by mycosporines, secondary metabolites that act as self-inhibitors of germination. The presence of nutrients, however, inhibits mycosporine biosynthesis and allows the germination of falcate conidia (Leite and Nicholson 1992). As a recent study revealed, mycosporines are not generated by oval conidia, making their germination independent of the presence of nutrients. Furthermore, nutrient depletion triggers germling fusion of oval conidia and the formation of melanized hyphopodia emerging from germling networks, enabling host tissue penetration. The availability of nutrients, however, represses the same processes in germlings derived from oval conidia (Nordzieke et al. 2019).

The nutritional factors required for spores to germinate are often overlooked, because the only cue needed for the spores of many pathogens to germinate is hydration. Nevertheless, this could be a future topic to study. Once the spores have germinated, enzyme activity, stomata, wounding of the leaf or pressure-generating appressoria allow penetration through the cuticle, epidermis and into the host, followed by invasive growth.

## *2. Initial penetration and interaction with the host*

Rice blast, *Magnaporthe oryzae*, is a model of choice to study fungal nutrient and developmental signaling during biotrophic growth and effector deployment in living host cells. The fungus *Magnaporthe oryzae* limits worldwide rice production (Wilson and Talbot 2009) and is one of only a few filamentous plant pathogens that directly threaten global food security (Fisher et al. 2012). To infect rice, specialized host-infecting cells (appressoria) develop at the tips of germ tubes emerging from *M. oryzae* spores attached to the leaf surface. Appressorium formation involves one round of mitosis, autophagic cell death of the spore, and the generation of enormous internal hydrostatic turgor. A penetration peg then emerges from the base of the mature appressorium, breaching the rice cuticle. In underlying epidermal cells, the penetration peg grows as a slender primary hypha that differentiates into branching invasive hyphae (IH). IH fill the first invaded cell before moving into adjacent living cells via plasmodesmata at around 44 hours post inoculation (hpi). *M. oryzae* initially grows undetected by the plant as a biotroph, colonizing successive living rice cells until the transition into necrotrophy around 96 hpi, when plant cells begin to die and necrotic lesions develop on the rice leaf surface from which new spores are produced to continue the lifecycle. To avoid triggering plant innate immunity during biotrophy, the fungus must neutralize the host oxidative burst that occurs even during a compatible interaction (Marroquin-Guzman et al., 2017; Segal and Wilson, 2018; Fernandez et al., 2014). Also, *M. oryzae* suppresses plant innate immunity by deploying cytoplasmic and apoplastic effectors into and between host cells during colonization. Cytoplasmic effectors like Pwl2 are deployed into rice cells via the highly focused and membrane-rich blast interfacial complex (BIC), which is located subapically between primary hyphae and IH in each invaded rice cell, while apoplastic effectors like Bas4 are secreted into the interfacial compartment between the fungal invasive hyphae (IH) and the plant-derived extra invasive hyphal membrane (EIHM) (Giraldo et al. 2013).

TOR is a conserved signaling pathway in eukaryotes that controls cell growth and development in response to nutrient sensing (Marroquin-Guzman et al. 2017b). Studies from Richard Wilson's lab in *M. oryzae* showed that inactive TOR signaling during spore germination on coverslips or rice leaf surfaces is required for preventing multiple rounds of mitosis that otherwise fail to induce autophagy and appressorium formation (Marroquin-Guzman et al., 2017b; Marroquin-Guzman and Wilson, 2015; Sun et al., 2019). Conversely, active TOR signaling is required immediately following penetration into rice cells in order to promote nuclear migration and mitosis and thus initiate *M. oryzae* biotrophic growth (Fernandez et al. 2014b). Whether TOR signaling interacted with effector deployment during biotrophy was not known, but recent investigations into TOR function in *M. oryzae* has revealed just such a connection. It was discovered how TOR signaling via a vacuolar membrane protein called Imp1 is essential for autophagy induction (Sun et al. 2018). Furthermore, Δ*imp1* deletants lacking a functional *IMP1* gene could form functional appressorium on rice leaf sheath surfaces, but following host invasion, Δ*imp1* biotrophic interface integrity became compromised over time. This abolished cytoplasmic effector secretion and the BIC, released apoplastic effectors into host cytoplasm and attenuated growth between rice cells. This loss of biotrophic growth and interface integrity did not occur due to early entry into necrotrophy, or due to impaired vacuole function, but rather resulted from altered membrane trafficking processes during in autophagy-impaired Δ*imp1* mutant strains. Specifically, using fluorescent markers and inhibitors, it was concluded that although Imp1 was required for fusing endosomes and autophagosomes to the vacuole, it was its additional role in supplying plasma membranes for phagophore expansion during autophagy induction that was critical for the longevity of the biotrophic interface and in maintaining effector deployment. Indeed, the effects of Δ*imp1* could be reversed, and biotrophic interface integrity and effector deployment restored, by the TOR-independent induction of autophagy. Conversely, preventing autophagy induction in wild type (after biotrophy was established) recapitulated the effector dysregulation, membrane erosion and loss of cell-to-cell movement observed for Δ*imp1* (Fig. 1). Thus, dynamic TOR status changes during biotrophy regulate biotrophic interface integrity and effector secretion in *M. oryzae* via autophagy. Our *IMP1* investigations unexpectedly linking fungal metabolism, effector secretion and fungal growth in - and between - rice cells thus provides new knowledge on the nature and regulation of the plant-fungal metabolic interface. When considered alongside work showing the importance of

autophagy-derived membranes in facilitating a eukaryote-prokaryote interaction between *Dictyostelium* and *Mycobacterium* (Gerstenmaier et al. 2015), our findings point to autophagy as a fundamental principle underlying interkingdom host-symbiont interactions. Once inside the rice leaf, *M. oryzae* elaborates pseudohyphal-like invasive hyphae that rapidly colonize living host cells, secreting effector molecules to suppress host immunity and facilitate infection. Hyphae then appear to locate pit fields, composed of plasmodesmata, which are traversed by constricted, narrow hyphae, enabling the spread of the fungus to adjacent host cells. The fungus rapidly colonizes host tissue, and disease lesions appear within 4 to 5 days of initial infection. Chemical genetic inhibition of a single fungal mitogen-activated protein (MAP) kinase, Pmk1, prevents *M. oryzae* from infecting adjacent plant cells, leaving the fungus trapped within a single plant cell (Sakulkoo et al. 2018). Pmk1 regulates expression of secreted fungal effector proteins implicated in suppression of host immune defenses, preventing reactive oxygen species generation and excessive callose deposition at plasmodesmata. Pmk1 MAPK pathway controls plant tissue invasion by controlling the constriction of invasive hyphae to traverse pit fields in order to invade new rice cells while maintaining the cellular integrity of the host. To accomplish this feat, the MAPK also regulates expression of a battery of effectors to suppress plant immunity, thereby preventing plasmodesmal closure until the fungus has invaded neighboring cells. Plant tissue invasion by the blast fungus is therefore orchestrated, rapid, and necessary for the devastating consequences of the disease.

Colonization is being addressed in other pathogens as well, for example *Fusarium oxysporum* where the Díaz-Mínguez lab is looking at the genetic basis of colonization and pathogenicity. Chromosome 14 is the smallest chromosome of the genome of *Fusarium oxysporum* (*F. oxysporum* f. sp. *lycopersici* strain 2487) and has been described as a "pathogenicity chromosome." It is one of the four complete chromosomes which constitute the lineage specific or adaptive genome of *F. oxysporum* (Ma et al. 2010). It contains loci that encode virulence/pathogenicity factors and confers pathogenicity to non-pathogenic strains after its transfer from a pathogenic strain. Also, it has been shown that complete loss of this chromosome results in the loss of pathogenicity, although partial deletions that affect only supercontig 22 do not reduce virulence (Vlaardingerbroek et al. 2016). This chromosome is likely equivalent to the smallest chromosome of *F. oxysporum* f. sp. *phaseoli* (FOP) strain FOP-SP1 as revealed by electrophoretic karyotypes. The *FTF* gene family is composed of two pathogenicity

factors: *FTF1*, with multiple paralogues all located in the small chromosome of highly virulent strains of FOP, and *FTF2*, a single copy factor located in the core genome. Both factors are involved in virulence/pathogenicity (Niño-Sánchez et al. 2016). Some strains carry a partial deletion of the small chromosome (FOP-SP1sChr-pΔ), as shown by the electrophoretic karyotypes analysis. Alignment of the complete sequence of one of the mutants with the wild-type genome (FOP-SP1) shows that missing regions in the mutant are spread in several contigs, and none of them fit with conserved chromosomes (core genome) in the wild-type genome. The deleted region includes all the paralogues of *FTF1*. Inoculation assays conducted on common bean plants demonstrate that FOP-SP1sChr-pΔ mutants show a complete loss of pathogenicity, suggesting that the genomic region missing in the mutants harbors the relevant genetic components required to produce disease in plants. Although the FOP-SP1sChr-pΔ mutant strains were unable to produce Fusarium wilt symptoms in infected common bean plants, confocal laser microscopic analysis revealed the ability of these strains to colonize the host, albeit to a less extent than highly virulent strains. These two facts show that the colonization phenotype of the mutant strains is very similar to that displayed by *F. oxysporum* endophytic isolates, suggesting that the deletion of the relevant region of the small chromosome is enough to turn a highly virulent strain into an endophyte-like strain.

## *3. Nutritional and metabolic factors contributing to the fungal attack following colonization*

When pathogens and pathosystems are considered together, one learns about the molecular decision-making processes and metabolic strategies underlying how fungi/oomycetes grow and develop in host cells, how fungi respond to their environment, how they acquire nutrients and utilize nutrients for growth and/or host defense suppression purposes, and how they neutralize host innate immunity and manipulate plant cell physiology to facilitate infection. The transcriptomic and metabolic response of the pathogen to the host environment, as an adaptive response, should provide the key to understanding how pathogens survive on and in the host, and the lifestyle. Biotrophic infection involves specialized hyphae - such as haustorial feeding structures, extracellular hyphae and intracellular - with roles in both trafficking plant defense-suppressing proteins called effectors into host cells and acquiring nutrients. A main focus is the contribution of primary carbon and nitrogen metabolism; primary metabolism; other topics that

were addressed as well include a strong focus on pH regulation, cation tolerance and discussion of redox and iron availability. All three of these latter processes, while not strictly metabolic, are critical elements of the biochemistry of the host-pathogen interface.

### *3.1. pH signaling*:

*Carbon regulation of environmental pH and its effect on pathogenicity and mycotoxin production*

pH influences most, if not all, processes of life. In fungi, ambient pH acts as a potent regulator of growth and development and fungal pathogenicity, and many saprophytic and pathogenic species have been shown to respond to local pH. More recently, pH has emerged as a key player in the control of fungal infections (Prusky and Wilson 2018). Infections caused by fungi are often associated with a pH shift, either alkalization by ammonia or acidification by organic acids (Prusky et al. 2013), in the surrounding host tissue. Ambient pH adaptation ensures the expression of the adequate set of genes at a given pH. This is crucial during fungal infection to ensure, for example, the correct deployment of virulence factors that function at a specific pH (Prusky et al. 2013). While the activation of the Pal/Rim pathway was shown to be essential for infection and gene activation in a number of fungal pathogens of humans, such as *Candida albicans* and *A. fumigatus* as well as the plant pathogens *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Alternaria alternata* and *Penicillium expansum.* The importance of nutritional factors effect on the modulation of fungal metabolism was less reported even if strongly contributes to the activation of PacC. It was reported that higher nutritional level of sugars contribute to higher acidification of the environment while the reduction in sugar content contribute to alkalization by ammonia. These studies have revealed new and unexpected ways by which fungi induce the activation of the transcription factor PacC during host alkalization to increase the fungal infections potential (Peñalva et al. 2008). The transcriptomic and metabolic response of the pathogen to the host environment, as an adaptive response, should provide the key to understanding how pathogens survive on and in the host, and the lifestyle.

*pH governs fungal pathogenicity through MAPK signaling*

Environmental pH is a critical factor controlling fungal growth and development. To survive and propagate in a dynamic pH environment, fungal pathogens have the capacity to sense and respond to environmental pH changes. Further, fungal pathogens also can change the

surrounding pH to increase their infectious potential (reviewed in (Fernandes et al. 2017). Although the mechanism of pH sensing and response is well studied in fungi, how exactly fungus-induced pH change contributes to pathogenicity is not fully understood. The invasive growth (IG) MAPK pathway is broadly conserved in fungi, and essential for infection in a wide range of plant pathogens (Turrà et al. 2014). A recent study in *F. oxysporum* revealed that extracellular alkalization triggers rapid phosphorylation of the IG MAPK, leading to enhanced virulence towards tomato plants (Masachis et al. 2016). However, the molecular events underlying pH-induced MAPK regulation were not clarified. It is increasingly appreciated that intracellular pH (pHi) acts as a general regulator of cellular functions such as growth and proliferation (Reshkin et al. 2014), life span (Hughes *et al*., 2012) and glucose response (Dechant et al. 2010). So far, the role of pHi in fungal infection has not been examined in detail. Using a *F. oxysporum* strain expressing the pH-sensitive GFP variant pHluorin, a rapid and transitory change in pHi was found in response to extracellular pH shift. Pma1, an essential plasma membrane $H^+$-ATPase, is the main regulator of pHi homeostasis in fungi (Kane 2016). Exogenous application of a specific inhibitor of Pma1 showed that a rapid and sustained decrease of pHi is able to modulate MAPK phosphorylation, supporting the idea that pHi acts as a key switch controlling MAPK activity. Understanding how pHi regulates MAPK signaling may reveal new ways to control fungal growth, development and pathogenicity.

*Cation-Stress-Responsive Transcription Factors SltA and CrzA Regulated Processes*

Using *Aspergillus nidulans* as a model, Espeso's lab analyzed the involvement of regulatory systems in the virulence of *Aspergilli*, *Colletotrichum* and *Penicillium*, especially those systems that allow the fungus to tolerate extreme changes in the environmental pH and high extracellular concentrations of cations. The PacC/Pal system has been of particular interest and studies conducted by different groups have highlighted this regulatory mechanism during the process of invasion of the host by different species of pathogens. The continuous study of the PacC/Pal system has allowed finding other regulatory mechanisms that participate in the tolerance to the alkaline pH (reviewed in Etxebeste and Espeso 2019), such as the calcineurin dependent transcriptional regulatory system mediated by the Crz factor or the Slt system in members of the Pezizomycotina (Spielvogel et al. 2008). The absence of Crz function greatly reduces the infective capacity of *Aspergillus fumigatus* (Soriani et al. 2008). Given the extreme dependence

of the Crz function on the calcineurin protein-phosphatase activity, the use of calcineurin inhibitors has been considered as an alternative treatment for fungal infections. The study of the Slt regulatory system is relevant given its restrictive phylogenetic distribution. The transcriptional factor SltA, which like PacC exists in the cell in different forms through a complex proteolytic processing, plays an essential role in the tolerance to the stress of excess of cations (Mellado et al. 2016). Recent studies on *C. gloeosporioides* have shown the role of both Crz and Slt systems in virulence (Dubey et al. 2016). The set of results shows the potential of these transcriptional regulatory systems to mediate the infective processes of multiple fungal species, with the incentive that the extraordinary specificity of some of its elements can be used as future targets of antifungals. The discovery of these different mechanisms of pH modulation adds a new example to the ongoing arms race between pathogen and host and may pave the way for further discoveries of cross kingdom pH regulation.

### *3.2. ROS and redox state*

Reactive oxygen species (ROS) are a key, early component of plant defense, and also provide signals to both plant and fungal cells. Virulence-related factors are all interconnected, and ROS and other redox-related metabolic factors play important roles during plant-pathogen interactions, including the ability of a fungus to sense and withstand the ROS produced by plant defenses. While many studies have examined ROS responses between specific plant-fungal interactions, questions remain about how these responses compare across different fungal pathogens.

Nicole Donofrio's team investigated the mechanisms of ROS generation and responses in the hemi-biotroph *Magnaporthe oryzae* and the necrotroph *Cochilobolus heterostrophus*, which cause rice blast and Southern Corn Leaf Blight, respectively. These experiments employed a genetically-encoded ROS sensor called HyPer, which is a circularly permutated YFP coupled to the OxyR transcription factor, to analyze the role of ROS during the infective life cycles of both fungi (Belousov et al. 2006; Huang et al. 2017). A HyPer line in *M. oryzae*, called Mo-HyPer, and a recently developed a HyPer line in *C. heterostrophus*, both show redox sensitivity as evidenced by hydrogen peroxide perfusion experiments on the conidia of each pathogen (Figure 2). Imaging these lines during infection on their respective hosts, barley and corn will help

determine distinct hotspots of ROS detection. Once identified for both fungi, it will be possible to screen randomly disrupted mutant libraries constructed in the HyPer background lines in order to identify fungal genes that aid in control of ROS production and detection during infection. Timing of ROS response, as well as genes recovered from the forward genetic screens will be compared between hemi-biotroph and the necrotroph. The movie in the following link (https://bit.ly/2tBrSQ7) displays recent progress with the Mo-HyPer line showing increased ROS levels during invasion of barley epidermal cells at 36 hours post inoculation. Further analysis of these lines will provide a unique insight into ROS during infection.

## *4. Nutritional factors modulating metabolism*

### *4.1. Nutritional factors and oomycete metabolism*

The nutritional strategies of pathogens range between the extremes of necrotrophy and biotrophy. The Judelson lab has been studying *Phytophthora infestans* and *Pythium ultimum* to learn how these lifestyles are reflected in pathogen metabolism (Judelson and Ah-Fong 2018; Ah-Fong et al. 2019). These species were selected since they belong to sister clades in the oomycete group, and cause important diseases on a common host, potato tubers. *Ph. infestans* is a hemibiotroph with an extended biotrophic stage, while *Py. ultimum* is necrotrophic throughout the disease cycle. Based on genome analysis, Judelson and coworkers observed that the metabolic capabilities of the two species were very similar, with only a few exceptions. Many metabolic genes belong to families that vary in size between the species, although RNA-seq indicated that the impact of family expansions is usually tempered by mechanisms that suppress the transcription of some family members (Ah-Fong et al. 2017). The expression levels of genes in many metabolic pathways were found to differ between the species during tuber infection. For example, the fraction of mRNA devoted to lipid, phytate, sulfate, and starch metabolism was greater in *Py. ultimum,* which can be explained by the fact that such nutrients mostly occur within plant cells and thus are inaccessible to *Ph. infestans* during biotrophic growth. Higher levels of expression of gluconeogenesis genes were observed in *Ph. infestans*, apparently due to the absence in that species of the starch-degrading enzyme γ-amylase, which is encoded by *Py. ultimum.* Also expressed at higher levels in *Ph. infestans* during biotrophic growth were pathways for amino acid biosynthesis, which reflects a need to make amino acids that occur at

limiting quantities in the apoplast. The transcription pattern of metabolic genes in *Ph. infestans* during late infection became more like that of *Py. ultimum,* consistent with the former's transition to necrotrophy. Divergence between the oomycetes were also seen in their incorporation of nitrate through the nitrate reductase pathway. Isotopic labeling and enzyme assays indicated that *Py. ultimum* makes greater use of nitrate. This was found to be due to the absence of a nitrogen metabolite repression transcription factor that in most other organisms serves to suppress the expression of nitrate reductase. The $K_m$ of that enzyme for nitrate was also much lower in *Py. ultimum.* These appear to be evolutionary adaptations that help *Py. ultimum* grow both as a pathogen on plants and as a saprophyte in soil. Gene silencing studies showed that nitrate reductase is nevertheless important to *Ph. infestans* when it grows on nitrate-containing tissues such as leaves, possibly to detoxify nitrate (Abrahamian et al. 2016). In summary, metabolic differences between the species could be attributed to differential access to nutrients, variation in gene content, and changes in the catalytic behavior of enzymes. The next step will be to integrate information about metabolism with studies of nutrient uptake, including the role of the specialized hyphae known as haustoria in feeding by *Ph. infestans* and whether that pathogen makes effectors that alter nutrient partitioning between plant cell and apoplast.

### *4.2. Secondary metabolism*

Deoxynivalenol (DON), a trichothecene mycotoxin produced by the wheat head blight fungus *Fusarium graminearum* is harmful to human and animals. As a potent inhibitor of protein synthesis in eukaryotic organisms, it is also an important virulence factor during plant infection. Nitrogen may also regulate secondary metabolites secreted by *Fusarium graminearum*. In this relation Jin-Rong Xu suggested that deletion of an ammonium permease from *F. graminearum* Ammonium and AreA play a critical role in regulating trichothecene biosynthesis in *F. graminearum*. DON production was suppressed by ammonium but stimulated by some nitrogen sources such as polyamines and arginine. The *areA* deletion mutant was defective in DON production and non-pathogenic (Hou et al. 2015). Deletion of AreA in *F. graminearum* also abolished the suppression of *TRI* gene expression by ammonium. Interestingly, *AREA* expression itself was suppressed by ammonium in this important wheat pathogen. Among three ammonium permease genes, *MEP2* appeared to play a more important role in ammonium sensing. It had the highest expression level at lower concentrations of ammonium. Deletion of *MEP2* resulted in the

expression of *AREA* and *TRI5* in the presence of 50 mM ammonium. The *mep2* deletion mutant also was defective in vegetative growth and plant infection. These results indicated that deletion of the *MEP2* ammonium permease may led to the loss or defects of ammonium sensing, which is important for regulating plant infection processes and DON production. However, the *mep2 mep3* double mutant had more severe defects in growth and plant infection than the single mutants. These two high affinity ammonium permease genes likely have distinct by overlapping functions in ammonium uptake in *F. graminearum*. As an ammonium sensor, the C-terminal tail region of Mep2 may interact with conserved intracellular signaling machinery to regulate *AREA* and *TRI* gene expression.

Differing from nitrogen response, (Maor et al. 2017) indicated as well the importance of carbon source, specifically sucrose, for the regulation of ochratoxin A (OTA) accumulation. The Sionov group, studying the effect of ambient pH modulation on ochratoxin A accumulation by *Aspergillus carbonarius* in grapes, suggested that the sucrose carbon source was able to modulate pH by induction of glucose oxidase (GOX) activity and gluconic acid accumulation resulting in enhanced levels of OTA accumulation and decay damage by *Aspergillus carbonarius* to grapes. The results indicate that high sugar concentrations favor high levels of organic acid production that result in a low final pH, strong induction of the OTA biosynthesis genes, and mycotoxin accumulation. Furthermore, increasing sucrose content was found to also positively impact expression of the global regulator of secondary metabolites, LaeA. An increased *laeA* expression was induced in high sucrose concentration (15%), which was reduced 7-fold in only 0.5% sucrose, suggesting that sugar concentration may play an important role as a regulator of OTA synthesis *in vitro* through induction of *laeA* expression. Deletion of *laeA* in *A. carbonarius* resulted in a drastic decrease in the OTA production and reduction in decay development in grape berries inoculated with *ΔlaeA* deletion mutant compared to the wild-type strain. The results indicate the importance of abiotic factors in LaeA regulation of OTA and other secondary metabolites that contribute to pathogenicity.

### *4.3. Carbohydrate metabolism*

*4.3.1. Crosstalk between the mitogen activated protein kinase SakAHOG1–MpkC and protein kinase A connects carbohydrate mobilization to cell wall biosynthesis*

418 The relation between nutritional factors and metabolic changes induced by pathogens can be
419 studied to great advantage in model species, here switching from the discussion so far of
420 agriculturally-relevant pathogens, to fundamental genetic work on a model Aspergillus pathogen
421 of humans, *Aspergillus fumigatus*. Aspergillus is an opportunistic human pathogen causing
422 allergic reactions or systemic infections such as invasive pulmonary aspergillosis, especially in
423 immune-compromised patients. In an example of principles that extend beyond plant pathogens
424 to virulence mechanisms in general, Goldman and co-workers indicated that *A.*
425 *fumigatus* mitogen-activated protein kinases (MAPKs) are involved in maintaining the normal
426 morphology of the cell wall and providing resistance against cell wall-damaging agents (Bruder
427 Nascimento et al. 2016). Upon cell wall stress, cell wall-related sugars are synthesized from
428 carbohydrate storage compounds. This process is dependent on cAMP-dependent protein kinase
429 A (PKA) activity and regulated by the high-osmolarity glycerol response (HOG) MAPKs SakA
430 and MpkC (de Assis et al. 2018). These protein kinases are necessary for normal
431 accumulation/degradation of trehalose and glycogen, and the lack of these genes reduces glucose
432 uptake and glycogen synthesis. Alterations in glycogen synthesis were observed for
433 the *sakA* and *mpkC* deletion mutants, which also displayed alterations in carbohydrate exposure
434 on the cell wall (de Assis et al. 2018). Carbohydrate mobilization is controlled by SakA
435 interaction with PkaC1 and PkaR, suggesting a putative mechanism where the PkaR regulatory
436 subunit leaves the complex and releases the SakA-PkaC1 complex for activation of enzymes
437 involved in carbohydrate mobilization (de Assis et al. 2018). This suggest that reduced
438 mobilization of monosaccharides for fungal cell wall biosynthesis during cell wall damage and
439 the osmotic stress response can causes defects in the structure of the fungal cell wall making
440 these pathways possible targets for new antifungal strategies. Elucidation of the cooperation
441 between the HOG and PKA pathways in the mobilization of carbohydrates for fungal cell wall
442 biosynthesis was reported. The reduced mobilization of simple sugars was suggested to cause
443 defects in the structure of the fungal cell wall. In summary, it was proposed that SakA is
444 important for PKA activity, therefore regulating the availability and mobilization of
445 monosaccharides for fungal cell wall biosynthesis during cell wall damage and the osmotic stress
446 response.
447

*4.3.2. Evolution of host range is associated with carbohydrate and protein metabolism in Colletotrichum spp.*

Thon's group brought a view of the fungal response to host carbohydrate and protein content, by the recent evolution of the *Colletotrichum acutatum* and *C. gloeosporioides* species complexes. Many carbohydrate active enzyme and protease encoding genes are present as large multicopy gene families, which may be the product of the evolution of diversity in gene expression patterns and enzyme substrate specificities. Comparison of carbohydrate active enzymes (CAZymes) and protease encoding gene families linked the relative expansion of these families to the host range showing a correlation between higher CAZyme and protease diversity and broader host range. Since the *C. acutatum* and *C. gloeosporiodes* species complexes are two evolutionarily divergent clades within the genus, two hypotheses may explain the observed patterns of gene family expansion: 1) the gene expansions occurred simultaneously during the evolution of the two species complexes and 2) the gene expansions were ancient and gene loss in the other *Colletotrichum* lineages explains their evolution of narrow host range, while the *C. acutatum* and *C. gloeosporioides* complexes maintained large gene families and broad host range. Subsequent phylogenetic analyses of the CE16: acetyltransferase and the M43B metallo-endopeptidase families (and others, not shown) revealed evidence of extensive gene loss in all lineages of *Colletotrichum* except the *C. acutatum* and *C. gloeosporioides* species complexes, consistent with hypothesis 2. These results also suggest that ancestral *Colletotrichum* species may have evolved as broad host-range pathogens and that host specificity is a relatively recent adaptation.

*Branched chain amino acid biosynthesis genes and their regulation in Aspergillus*

The molecular genetics of biosynthesis of the branched chain amino acid (BCAA) leucine was reported for *Aspergillus nidulans* during in vitro growth. Two genes encode beta-isopropylmalate dehydrogenases (β-IDHs), and six genes potentially encode BCAA aminotransferases (BATs), the enzymes for the last two biosynthetic steps. Todd's group demonstrated that simultaneous deletion of both β-IDH genes is needed to generate a tight leucine auxotroph, indicating that both genes function in leucine biosynthesis. The BATs, in addition to their role in the last step of leucine biosynthesis, catalyze the final biosynthetic step of the BCAAs isoleucine and valine, as well as the first step in degradation of all three BCAAs. Deletion of none of the six BAT genes confers BCAA auxotrophy. However, combinations of double mutants revealed that the two most highly expressed BAT genes function in BCAA biosynthesis as well as in their

degradation. Two other BATs, which are encoded within the aspercryptins biosynthetic gene cluster, are responsible for biosynthesis of unusual BCAAs that are precursors for the biosynthesis of the aspercryptins family of secondary metabolites (Chiang et al. 2016; Henke et al. 2016). The transcription factor LeuB was thought to regulate leucine biosynthesis, as the *leuB* deletion mutant is a partial leucine auxotroph (Downes et al. 2013). LeuB was shown to activate expression of the leucine biosynthesis pathway genes. The leucine biosynthesis pathway represents an example of gene duplication and neofunctionalization. In *M. oryzae* and *F. graminearum,* BCAA biosynthesis genes are involved infection-related morphology and virulence (e.g. Du et al. 2013; Liu et al. 2015; Patkar et al. 2012). Therefore, the underlying molecular genetics of fungal BCAA metabolism and, in particular, the role of duplicated genes within this pathway in different fungi are relevant to pathogenesis.

## *5. Nutritional factors and host metabolism*

Investigating the *Colletotrichum higginsianum* - *Arabidospis* pathosystem, Voll and co-workers found that carbon shortage at night impairs SA-dependent defense (Engelsdorf et al. 2013) , while conversely, increased sugar levels in the *sweet11/sweet12* double mutant result in a stronger SA response (Gebauer et al. 2017). Systematic starvation experiments showed that carbohydrate supply by the host is dispensable during biotrophic growth of *C. higginsianum*, while carbon deficiency was most harmful to the host during the necrotrophic colonization phase (Engelsdorf et al. 2013). Compared with the wild type, the increases in the total salicylic acid pool and the phytoalexin camalexin accumulation were reduced in starch-free mutants at late interaction stages. During the early interaction, however, an increased free salicylic acid pool did not convey elevated pathogenesis-related gene expression in starch-free mutants. These observations suggest that reduced carbon availability dampens induced defense responses in Arabidopsis.

In this same perspective, Voll and Gebauer analyzed the localization of translational *AtSWEET12-YFP* reporter gene fusions during powdery mildew infection. *AtSWEET12* is the only *SWEET* (SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER) that was reported to be induced in the interaction with powdery mildew (Chen et al. 2010). Since powdery mildew establishes in the epidermis by forming specialized hyphae that serve the uptake of organic building blocks from the host (Sutton et al. 1999; Fotopoulos et al. 2003), the finding by

Chen et al. (Chen et al. 2010) had led to the hypothesis that At*SWEET12* might accumulate in the extra-haustorial membrane to provide sucrose to the encased fungal haustoria. However, localization of the reporter gene fusions in powdery mildew infected leaves (Fig. 3 A,B) indicated that *At*SWEET12-YFP did not accumulate in the extrahaustorial membrane, suggesting that it does not provide sucrose to powdery mildew haustoria. Observations indicated that the fluorescence reporter accumulates in the leaf vasculature upon powdery mildew infection, and might be engaged in modulating phloem loading in infected leaves.

### *6. Effectors*

Secreted effectors are a key part of fungal-plant interactions, alongside metabolic factors. Great progress in linking effectors and metabolism has been made in the *Ustilago maydis* – maize interaction. Regine Kahmann gave the closing note of the workshop with a view of the core effectors, showing how a transcription factor (NLT1) associated with tumor formation regulates a set of effector genes belonging to the tumor formation cluster (Lanver et al. 2018).

### *7. Intracellular traffic*

Miguel Peñalva noted that for a number of model examples, the ability of plant pathogenic fungi to infect their hosts has arrived at a sufficient degree of understanding to explain plant-pathogen interactions in cell biological terms. Plant pathologists may now take advantage of the current understanding of intracellular traffic that has been achieved using model fungi such as *Neurospora crassa* and *Aspergillus nidulans*.

## Conclusions

The focus of this meeting was to analyze how the lifestyles and nutritional strategies of eukaryotic filamentous phytopathogens are related to the metabolic architectures and pathogenic processes affecting both host and pathogen. The differential virulence of pathogens in senescing tissue with dynamic nutritional changes has been addressed in depth in plant pathology. The activation of pathogenicity factors, including fungal penetration, activation of pathogenicity

factors (elicitors, enzyme secretion, etc.), colonization and corresponding induced senescence of the tissue were not compared under differential nutritional factors in leaf tissue. However the differential virulence levels observed in fruit and vegetable tissue by pathogens as observed during ripening resulting in increased nutritional availability and senescence give rise to several questions about the importance of these changes in pathogenicity. The question is if either the occurrence of nutritional availability or the differential senescing of leaves affect pathogen attack on the leaf. How, then, does the metabolic regulation by fungi occur during the transformation from biotrophic/quiescent to active infections? When and how do the fungal systems switch from their own stored nutritional factors to those induced in the host? What are the signals that activate fungal primary metabolism to produce the initial molecules contributing to fungal pathogenicity? What differential metabolism is activated to acquire nutrients from the developing host tissue vs. the fully mature one? Transcriptional, biochemical, and functional analyses of fungal genes in biotrophic and hemibiotrophic foliar pathogens and necrotrophic fruit pathogens have attempted to answer these questions, and to characterize the contribution of fungal metabolism during plant infection.

How internal nutritional factors or host physiology can modulate the formation of macro- and microconidia of foliar leaf pathogens is not known. In *M. oryzae* however, the transition of biotrophy into necrotrophy starts when plant cells begin to die and necrotic lesions develop on the rice leaf surface from which new spores are produced to continue the lifecycle. The question is how host cell viability or senescence and the released nutritional factors signal the activation of effector deployment during biotrophy. The facts are that the pseudo-hypha like invasive hyphae that rapidly colonize living host cells and secrete effector molecules to suppress host immunity are located close to the plasmodesmata. Through this structure a range of nutritional molecular cargoes of different sizes are transferred. These may contribute to the transverse penetration of the constricted hyphae and contribute to fungal colonization. Nutrient metabolic differences between oomycete species such as the differential carbon metabolism or those observed in fruit pathogens that contribute to pH modulation, may also affect the variation in gene expression and enzyme catalytic behaviour. This may suggest the importance of nutrient availability. Nutrients may affect, as well, the differential ROS- response of hemi-biotrophs and necrotrophs. Environmental conditions may as well be critical for the differential cell viability and/or nutritional availability that could be considered. On the other side the reduced

mobilization of simple sugars in Aspergillus by SakA for PKA activity, may cause defects in the structure of the fungal cell wall resulting in damage and the osmotic stress response. These findings in different systems may indicate that the process of fungal signalling may be regulated by the dynamics of nutritional availability in the host.

Nutritional factors such as nitrogen may also modulate secondary metabolic processes, affecting, for example, the secretion of mycotoxins by *Fusarium graminearum*. In Aspergillus, sucrose may regulate secondary metabolite accumulation, for example ochratoxin A. The availability of sugar in the plant environment, however, may differentially modulate alkalization or acidification of the tissue environment via the secretion of ammonia or organic acid, respectively. These pH signals contribute to the gene activation of pathogenicity factors (Prusky et al. 2004). Furthermore, recent findings indicate that pathogens can either alkalize or acidify the host environment (Bi et al. 2016). These findings are of high biological relevance, because pathogens are likely to encounter different levels of carbon availability, depending on the host niche (biotrophic) or the mechanism of infection (necrotrophic). Furthermore the intracellular pH (pHi) acts as a general regulator of cellular functions such as growth and proliferation (Reshkin et al. 2014). These metabolic changes may contribute to gene expression, differential carbohydrate active enzyme activities, MAPK pathway activation and the tolerance to the adaptation to changes in the environmental pH and high cation concentrations during colonization.

While sugar levels may modulate fungal metabolism and pH changes, systematic starvation experiments showed that carbohydrate supply by the host is dispensable during biotrophic growth of *C. higginsianum*, while carbon deficiency was most harmful to the host during the necrotrophic colonization phase (Engelsdorf et al. 2013). Furthermore, mechanisms of resistance and accumulation of the phytoalexin camalexin were reduced in starch-free mutants at late interaction stages. All these together indicate that the understanding the genetic pathway and the consequent metabolic processes in pathogenic fungi that modulate their environment is paramount to developing effective disease-prevention strategies (Fernandes et al. 2017; Vylkova 2017). This indicates a need to understand the consequences of nutrient availability in fruits, as well as the daily variation of nutrients in leaves and the consequent expression of genes that modulate fungal activation and colonization.

A new metabolism-based strategy derives from recent work presented in the workshop by the Covo group, on the central cofactor NAD+. The control and maintenance of NAD+ homeostasis is needed for viability, making the NAD+ biosynthetic pathway, and its link to redox homeostasis, a target for antifungal development. This strategy was directly addressed in *Fusarium oxysporum*, where it was shown that nicotinaldehyde (NA), which inhibits a key enzyme in the salvage pathway of NAD+ biosynthesis, caused reductive stress and suppressed growth (Anand et al. 2019). Future work should lead to many more such approaches.

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FIGURE LEGENDS

**Figure 1**. Signaling vs nutritional factors. Examples are shown from studies on the *Magnaporthe oryzae* rice pathosystem, showing the parallel between (A) nutritional factors (Sun et al. 2018) and (B) classic signaling (from Sakulkoo et al., 2018; with permission). In (A), the scale bar indicates 10 μm, arrow indicates penetration sites, * indicates cell-to-cell movement; 3-MA is treatment at 36 hpi with the autophagy inhibitor 3-methyladenine; AM is treatment at 36 hpi with the TOR independent autophagy stimulant amiodarone. In (B) the bar is 20 μm and * indicates penetration sites.

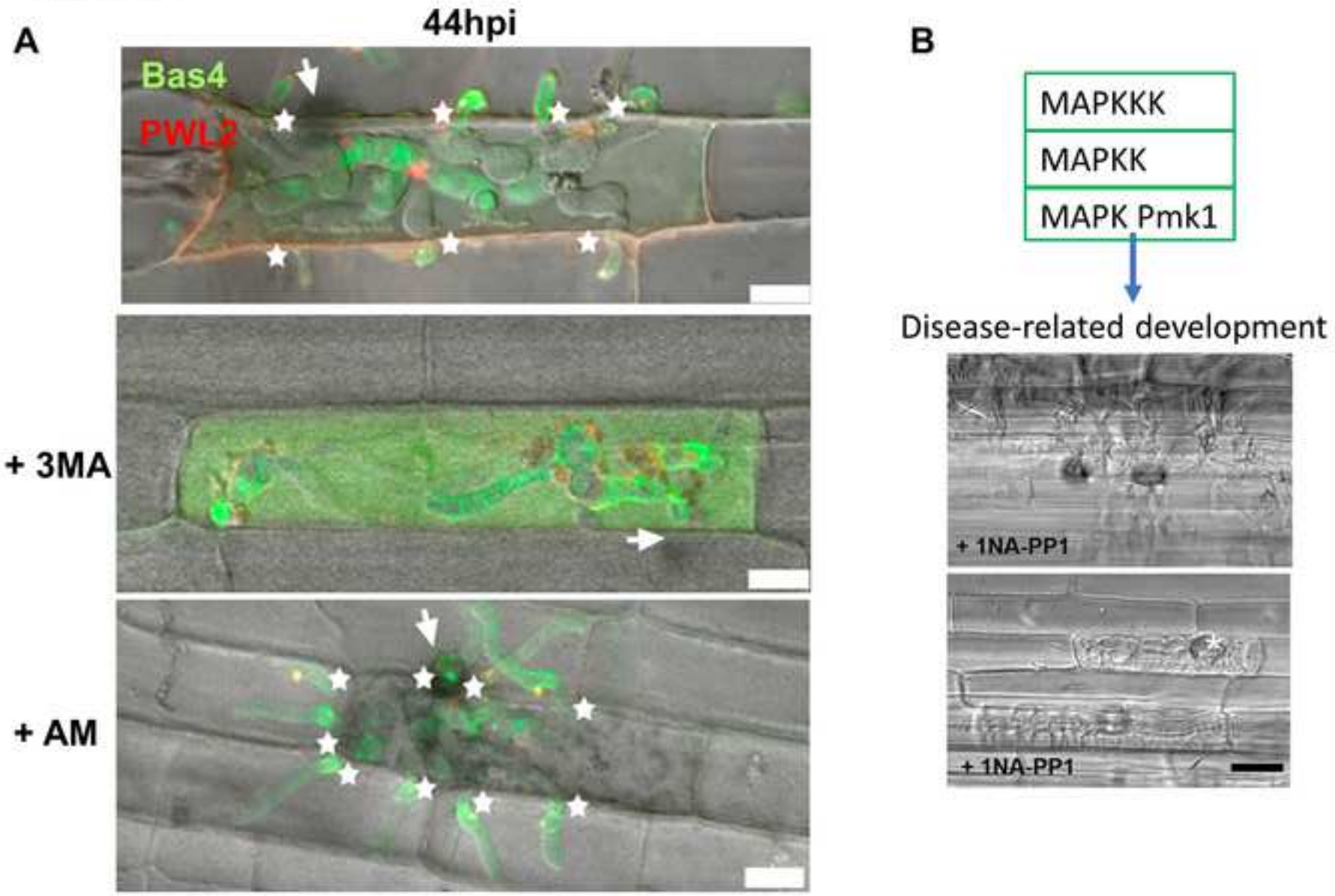
**Figure 2**. A genetically encoded reporter of reactive oxygen species (ROS). Conidia of *C. heterostrophus* (left two panels) and *M. oryzae* (right two panels) were harvested at 7 days old, pipetted onto a coverglass and inserted into a Chamlide Open Perfusion Chamber. The conidia were washed in sterile $H_2O$ (blue), 1 mM dithiotreitol (DTT) (yellow) and 10 mM $H_2O_2$ (pink). Graphs show HyPer emission ratio of 405:516. Rainbow bars on the images indicate intensity of ROS detection, with warmer colors indicating higher ROS. Imaged on a Zeiss 710 confocal, 63X NA 1.4 oil.

**Figure 3**. Induction and localization of *pAtSWEET12*:*At*SWEET12-YFP upon *E. cruciferarum* infection in the vasculature.

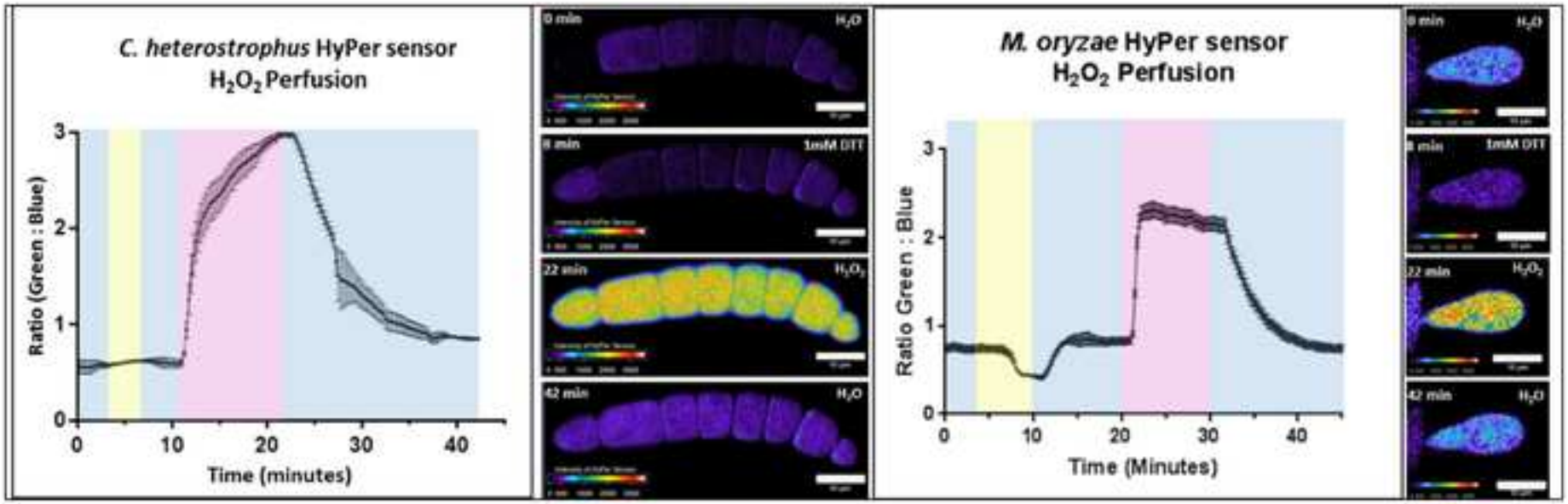
A) Fluorographs at binocular resolution were taken from control (top row) and *E. cruciferarum* infected plants (bottom row) 5 days after inoculation with 68 conidia $mm^{-2}$ at a stage in which infected leaves were covered with a dense epiphytic mycelium. The scale bar indicates 1 mm.

B) CLSM analysis of *pAtSWEET12*:*At*SWEET12-YFP (upper two rows) and Col-0 (lower two rows) at 7 dpi with *E. cruciferarum*. Channels are indicated above the respective columns. Two representative specimens are shown each, with the upper showing an overview of epidermis cells bearing compact haustoria, and the lower depicting a closeup of one compact *E. cruciferarum* haustorium. White arrows indicate the position of haustoria. The scale bar represents 25 μm.

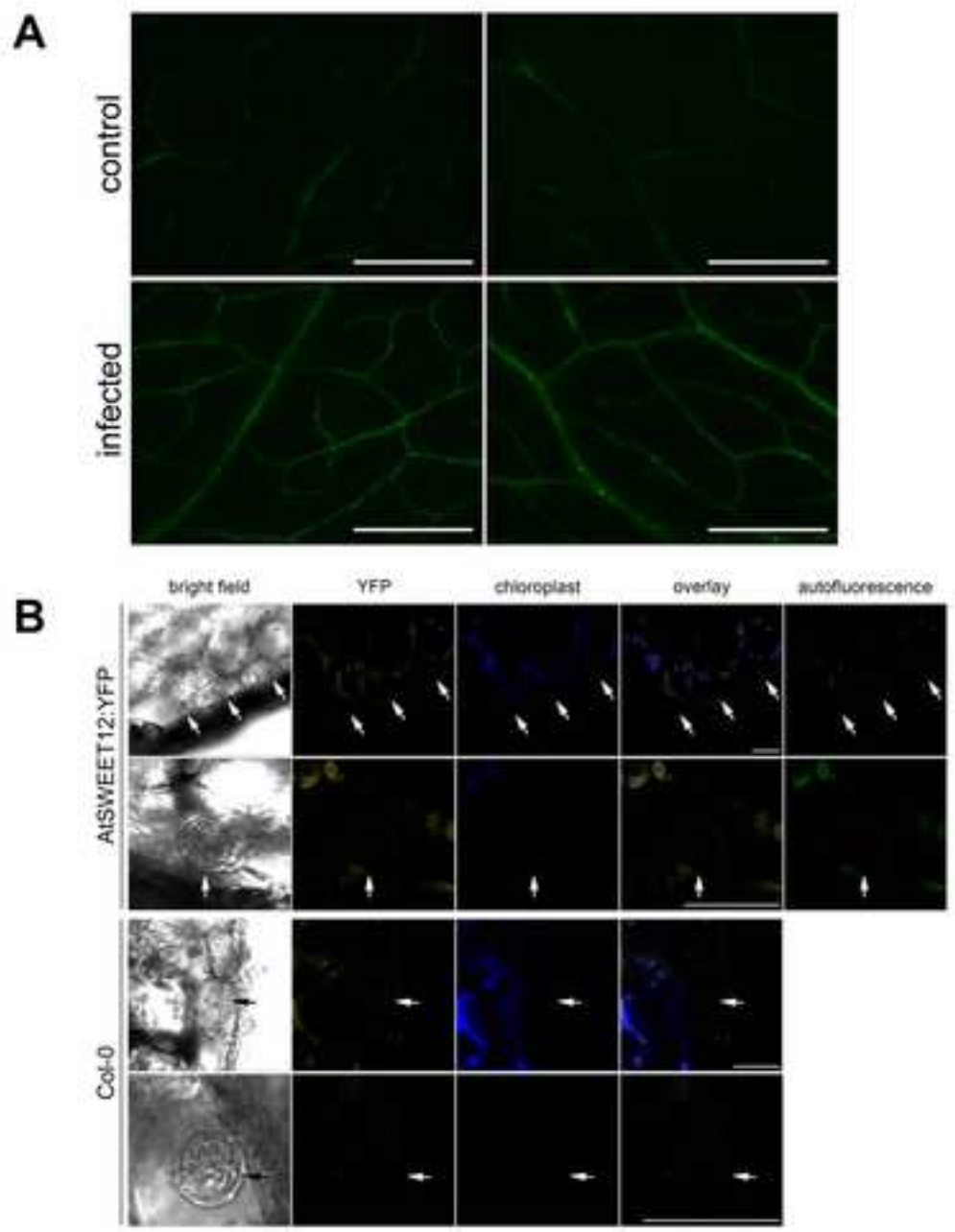
# Figure 1


A
44hpi
Bas4
PWL2
+ 3MA
+ AM
B
MAPKKK
MAPKK
MAPK Pmk1
Disease-related development
+ 1NA-PP1
+ 1NA-PP1

# Figure 2


C. heterostrophus HyPer sensor
H2O2 Perfusion
Ratio (Green : Blue)
Time (minutes)
0 min
H2O
8 min
1mM DTT
22 min
H2O2
42 min
H2O
M. oryzae HyPer sensor
H2O2 Perfusion
Ratio Green : Blue
Time (Minutes)

# Figure 3


A
control
infected
B
bright field
YFP
chloroplast
overlay
autofluorescence
AtSWEET12:YFP
Col-0