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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

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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.

Keywords Nutritional factors · Fungal · Magnaporthe · Aspergillus · Fusarium · PH sensor · Intracellular redox state · Workshop report 2018 · Carbon metabolism · Nitrogen metabolism

Nutritional factors are central to the molecular dialog between fungal pathogens and their plant hosts. In fruit pathogens, for example, infections remain quiescent until the fruit matures 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 (Prusky and Wilson 2018). Perhaps less obvious, nutritional factors are involved throughout fungal-plant interactions, even when the host plant is not undergoing a major metabolic change. Indeed, the distinction between a signal and a nutritional factor is often merely one of substrate concentration. Sub-micromolar 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 a case in point, consider the initial biotrophic phase of rice blast infection (Fig. 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 h 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. 1 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. 1, middle panel). Stimulating autophagy with amiodarone treatment increases cell-to-cell movement (Fig. 1, bottom panel). Thus, autophagy, and its control in response to the metabolic status of the fungal cell (Sun et al. 2018) is central to the biotrophic growth stage before symptom development. 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 (Wilson et al. 2007; 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 *M. oryzae* for growth in a glucose-rich, nitrogen-limiting environment (Fernandez et al. 2014c).

Conserved cell signaling pathways of fungi have been studied intensively in the past few decades. These pathways are central to development of the rice blast pathogen on the host. Sakulkoo et al. (2018) recently showed, with an allele-specific inhibitor, that invasive movement from the first infected cell to neighboring cells cannot occur in the absence of signaling through the Pmk1 MAP kinase pathway (Fig. 2). Pmk1 signaling is needed to open the route through plant plasmodesmata and for secretion of effectors (Sakulkoo et al. 2018). How this pathway might crosstalk with the TOR-autophagy pathway is not known,

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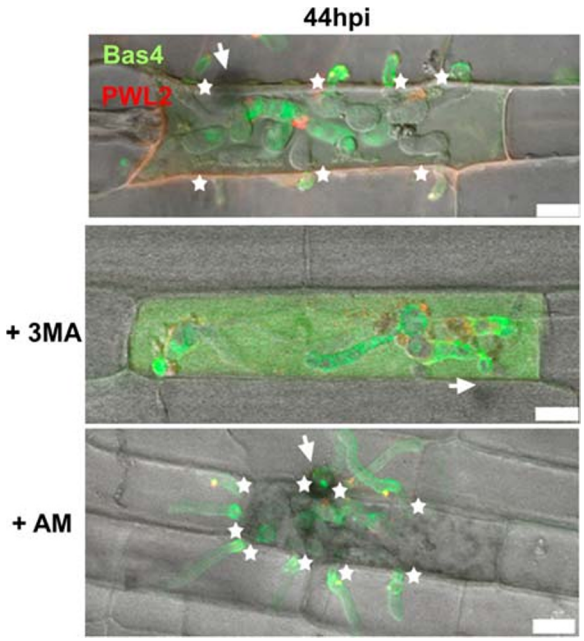

Fig. 1 Fungal metabolic status governs *Magnaporthe oryzae* biotrophic behaviour in rice cells. *Top panel* following host invasion, *M. oryzae* elaborates bulbous invasive hyphae (IH) in the first infected rice cell, growing in intimate contact with the living host surrounded by plant-derived membranes that form an interfacial compartment into which apoplastic effectors can be secreted. In the untreated top panel, the *M. oryzae* IH is outlined by the apoplastic effector Bas4 - visible after confocal microscopy because this otherwise wild type strain is expressing Bas4 fused to green fluorescent protein - indicating that the biotrophic interface is intact. The images are taken at 44 h post inoculation (hpi) when, in the top panel, IH is moving to adjacent cells (indicated by stars). In addition to Bas4-GFP, this strain also expresses Pwl2 fused to mCherry. Pwl2 is a cytoplasmic effector that accumulates in the focal, plant membrane-derived biotrophic interfacial complex (BIC). *Middle panel* blocking autophagy induction at 36 hpi by treatment with the autophagy inhibitor 3-methyladenine (3-MA) prevents IH in the first infected cell from moving to adjacent cells and, moreover, results in loss of the biotrophic interface, as revealed by release of Bas4-GFP into the cytoplasm. *Bottom panel* treatment of the first infected rice cell with the autophagy stimulator amiodarone (AM) at 36 hpi results, by 44 hpi, in the enhanced movement of IH to adjacent cells. Panels are taken from Sun et al. (2018). Because autophagy is induced under starvation conditions by the conserved Target of Rapamycin (TOR) nutrient signaling pathway, the conclusion drawn from these results is that autophagy induction both stimulates biotrophic growth to adjacent cells, and is required for maintaining the biotrophic interface, thus connecting metabolic signaling by TOR to autophagy-dependent interface integrity and growth in rice cells. The scale bars indicate 10 μm; arrows indicate penetration sites

but the comparison of nutritional factors and cell signaling underscores the importance of both, as well as the complexity of fungal-host interactions.

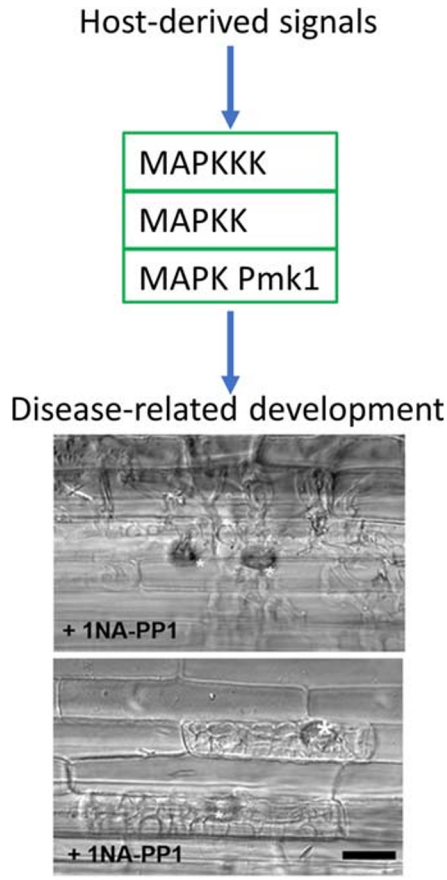

Fig. 2 *Magnaporthe oryzae* biotrophic invasion of rice cells depends on protein kinase signaling. Pmk1 (Pathogenicity-related MAP kinase), belonging to a conserved MAPK kinase cascade shown in the diagram, is required for plant cell-to-cell invasion (from Sakulkoo et al. 2018, with permission; presented at the workshop). Highly specific inhibition of Pmk1 in an analog-sensitive mutant (lower panel) has blocked invasion of neighboring cells, while the wild-type (upper panel) develops normally. The bar is 20 μm. 1NA-PP1 (1-naphthyl-PP1) is the kinase-inhibitory ATP analog 1-(Tert-Butyl)-3-(naphthalen-1-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine. * indicates an appressorium penetration site; the appressorium itself is visible as a shadow above the focus of the image. Images show development at 48 hpi following treatment with 5 μM 1NA-PP1 at 26 hpi

With these and other themes in mind, 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. 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.

Sporulation and germination

Fungal plant diseases are often propagated by spores, and most infection cycles begin when spores land on a suitable host and germinate. Fungi often have more than one spore type, reflecting, perhaps, adaptations to different stages of the infection cycle. For example, several ascomycetous fungi generate morphologically distinct asexual conidia. This includes saprotrophic species like *Neurospora crassa*, but also plant pathogenic fungi like *Colletotrichum graminicola* and *M. oryzae* (Panaccione et al. 1989; Kato et al., Kato and Mayama 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 distinct conidia types within infection and life cycles.

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 to distribute the pathogen via the vascular system. Since microconidia are able (albeit less efficiently) to cause rice blast symptoms, they could have roles in pathogenicity (Zhang et al. 2014). Also the corn anthracnose fungus *C. graminicola* generates two types of asexual conidia on distinct sites of infected plants: the smaller oval conidia are constricted from hyphae in the vascular system whereas the larger falcate conidia are borne from short conidiophores in asexual fruiting bodies on the plant surface (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 (Nordzieke et al. 2019). Furthermore, nutrient depletion triggers oval conidia germling fusion and the formation of melanized hyphopodia emerging from germling networks. This enables host tissue penetration. The availability of nutrients, however, represses the same processes (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, keeping in mind the function of multiple spore types.

Initial penetration and interaction with the host

The rice blast fungus, *M. oryzae*, has recently emerged as a tractable system to study fungal nutrient responses and developmental signaling during biotrophic growth and effector deployment in living host cells (Sakulkoo et al. 2018; Sun et al. 2018). *M. 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, *M. oryzae* spores attached to the leaf surface germinate and develop specialized host-penetration cells (appressoria) at the tips of germ tubes. 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 and the underlying epidermal cell wall. The penetration peg grows by invagination of the host cell membrane 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 h 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. 2017a; Segal and Wilson 2018; Fernandez et al. 2014a). 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 in *M. oryzae* by the Wilson lab 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 such a connection. TOR signaling, via a vacuolar membrane protein called Imp1, is now known to be essential for autophagy induction (Sun et al. 2018). Mutants lacking a functional *IMP1* gene could form functional appressoria on rice leaf sheath surfaces, but following host invasion, Δ*imp1* biotrophic interface integrity became compromised over time. This abolished the BIC and cytoplasmic effector secretion, released apoplastic effectors into the 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 played an additional role in supplying plasma membranes for phagophore expansion during autophagy induction. This was determined to be 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. These *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), the 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. Workshop attendee Nick Talbot discussed how 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 plant pathogens as well. One example is *Fusarium oxysporum*, where the Díaz-Mínguez lab studies the genetic basis of colonization and pathogenicity. Chromosome 14 is the smallest chromosome within the *Fusarium oxysporum* (*F. oxysporum* f. sp. *lycopersici* strain 2487) genome and has been described as a "pathogenicity chromosome." It is one of the four complete chromosomes that constitute the lineage specific or adaptive genome of *F. oxysporum* (Ma et al. 2010). Chromosome 14 contains loci that encode virulence/pathogenicity factors, which confer pathogenicity to non-pathogenic strains after their transfer from a pathogenic strain. Importantly, research has demonstrated 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 across 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 findings demonstrate 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.

Nutritional and metabolic factors contributing to invasive growth following surface colonization

When pathogens and their hosts are considered together, one learns about the molecular decision-making processes and metabolic strategies underlying how fungi/oomycetes grow and develop in host tissues, 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 adaptive transcriptomic and metabolic responses of the pathogen to the host environment should provide a key to understanding how pathogens survive on and in the host. A major emphasis at the workshop was the contribution of primary carbon and nitrogen metabolism; other topics that were addressed 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 and affect the ability of the pathogen to acquire and metabolize host compounds.

pH signaling

As presented by members of the Prusky, Di Pietro, Espeso and Peñalva labs, local environmental pH is an important signal. To survive and propagate in a dynamic pH environment, fungal pathogens have evolved 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). In fungi, ambient pH acts as a potent regulator of growth, development and pathogenicity. Many saprotrophs and pathogens 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, in the surrounding host tissue (Prusky et al. 2013). 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). The activation of the Pal/Rim pathway is 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*.

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

In contrast to pH regulation, there have been fewer reports on nutritional factors that modulate fungal metabolism, despite their strong contribution to how the pathogen controls local pH. Recent research implicates carbon availability in the host environment as a key factor triggering production and secretion of small pH-modulating molecules by the pathogen: ammonia and organic acids. Thus, high sugar levels promote acidification of the environment by gluconic acid, while lower sugar content promotes alkalization by ammonia (Bi et al. 2016). pH studies of plant pathogenic fungi have, indeed, revealed new and unexpected ways by which fungi induce the activation of the pH-sensitive transcriptional activator PacC during host alkalization to increase the potential of the pathogen to cause infection (Peñalva et al. 2008).

pH governs fungal pathogenicity through MAPK signaling

Although the mechanism of pH sensing and response is well-studied in fungi, it is not fully understood how fungus-induced pH changes contribute to pathogenicity. This question is being addressed by the Di Pietro group. 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). The molecular events underlying pH-induced MAPK regulation were, however, 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 and Gottschling 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 can 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, the Espeso lab analyzed how the regulatory systems that provide tolerance to large changes in ambient pH and cation levels are involved in the virulence of *Aspergilli*, *Colletotrichum* and *Penicillium*. 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 pathogen species. Studies of the PacC/Pal system have continuously led to the discovery of other regulatory mechanisms that participate in the tolerance to alkaline pH (reviewed in Etxebeste and Espeso 2019). These include 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 Crz function on the protein-phosphatase activity of calcineurin, 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. Transcriptional regulator SltA, like PacC, exists in the cell in different forms resulting from complex proteolytic processing.

SltA plays an essential role in the tolerance to the stress of excess 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 data show the potential of these transcriptional regulatory systems to mediate the infection processes of multiple fungal species. The generality of these regulatory pathways suggests that the extraordinary specificity of some of the elements involved can be used as future targets for antifungals. Furthermore, 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.

ROS and redox state

Reactive oxygen species (ROS) are a key, early component of plant defense, and 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.

The Donofrio group investigated the mechanisms of ROS generation and responses in the hemi-biotroph *M. 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 yellow fluorescent protein (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 MoHyPer, and a recently developed HyPer line in *C. heterostrophus*, both show redox sensitivity as evidenced by hydrogen peroxide perfusion experiments on the conidia of each pathogen (Fig. 3). Imaging these lines during infection on their respective hosts, barley and corn, will help determine distinct areas of increased ROS levels. 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 the hemi-biotroph and the necrotroph. A movie (Supplementary data) displays recent progress with the MoHyPer line showing increased ROS levels during invasion of barley epidermal cells at 36 h post inoculation. Further analysis of these lines will provide a unique insight into ROS during infection.

NAD^+ is a key molecule in redox state determination, functioning in ROS metabolism, along with its many other roles. A new metabolism-based strategy derives from recent work presented in the workshop by the Covo group, on this central cofactor (NAD^+). 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 the discovery of more roles for NAD^+ metabolism in host infection.

Nutritional factors modulating metabolism

Nutritional factors and oomycete metabolism

The nutritional strategies of pathogens range between the extremes of necrotrophy and biotrophy. The Judelson lab has been studying the oomycetes *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. *P. infestans* is a hemibiotroph with an extended biotrophic stage, while *P. 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

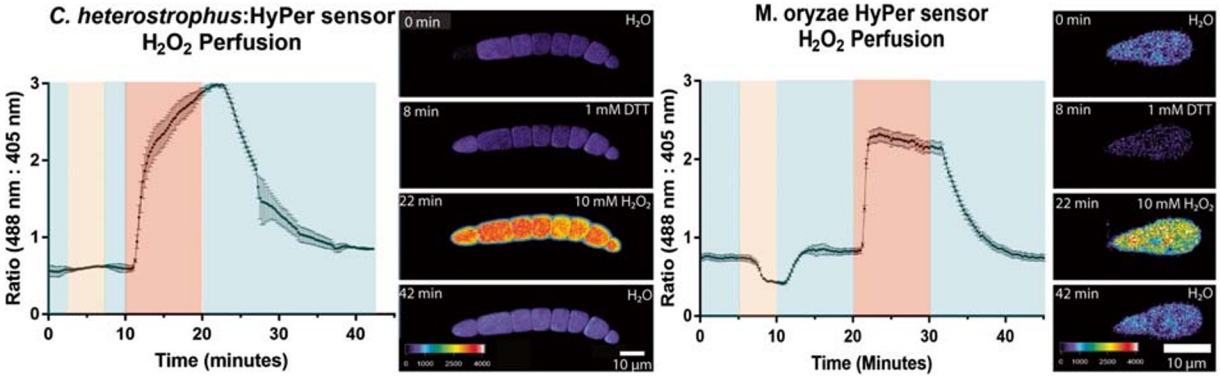

Fig. 3 A genetically encoded reporter of reactive oxygen species (ROS). Conidia of *C. heterostrophus* (left two panels) and *M. oryzae* (right two panels) were harvested at 7 days old, pipetted onto a coverglass and inserted into a Chamlide Open Perfusion Chamber. The conidia were washed in sterile 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

mRNA devoted to lipid, phytate, sulfate, and starch metabolism was greater in *P. ultimum,* which can be explained by the fact that such nutrients mostly occur within plant cells and thus are inaccessible to *P. infestans* during biotrophic growth. Higher levels of expression of gluconeogenesis genes were observed in *P. infestans*, apparently due to the absence in that species of the starch-degrading enzyme γ-amylase, which is encoded by *P. ultimum.* Also expressed at higher levels in *P. 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 *P. infestans* during late infection became more like that of *P. ultimum,* consistent with the former's transition to necrotrophy. Divergence between the oomycetes was also seen in their incorporation of nitrate through the nitrate reductase pathway. Isotopic labeling and enzyme assays indicated that *P. 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 *P. 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 *P. 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 *P. infestans.* Additionally, an important question will be whether the pathogen makes effectors that alter nutrient partitioning between plant cell and apoplast.

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*. Addressing this question, Jin-Rong Xu's group studied loss of function mutants and found that an ammonium permease from *F. graminearum* and the transcriptional regulator AreA play a critical role in regulating trichothecene biosynthesis. 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 was non-pathogenic (Hou et al. 2015). Deletion of AreA in *F. graminearum* also abolished the suppression of trichothecene biosynthesis gene (*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 lead to the loss or defects of ammonium sensing, which is important for regulating plant infection processes and DON production. The *mep2 mep3* double mutant, however, had more severe defects in growth and plant infection than the single mutants. These two high affinity ammonium permease genes likely have distinct but 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.

Secondary metabolism is also regulated by the carbon source. Studies presented by the Sionov group highlighted the importance of sucrose for the regulation of ochratoxin A (OTA) accumulation (Maor et al. 2017). Studying the effect of ambient pH modulation on ochratoxin A accumulation by *Aspergillus carbonarius* in grapes, they found evidence that the sucrose carbon source was able to modulate pH by induction of glucose oxidase (GOX) activity. Gluconic acid accumulation resulted in enhanced levels of OTA accumulation and decay damage by *Aspergillus carbonarius* to grapes. As noted above, high sugar concentrations favor high levels of organic acid production. This results in a low final pH, strong induction of the OTA biosynthesis genes, and mycotoxin accumulation. Furthermore, increasing sucrose content was found to positively impact expression of the global regulator of secondary metabolite biosynthesis, LaeA. Increased *laeA* expression was observed at high sucrose concentration (15%), which was reduced seven-fold in 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 OTA production and reduction in decay development in grape berries inoculated with the mutant compared to the wild-type strain. The results indicate the importance of nutrient factors in LaeA regulation of OTA and other secondary metabolites that contribute to pathogenicity and toxicity.

Carbohydrate metabolism

Crosstalk between the mitogen activated protein kinase SakAHOG1–MpkC and protein kinase a connects carbohydrate mobilization to cell wall biosynthesis

The relation between nutritional factors and metabolic changes induced by pathogens can be studied to great advantage in model species. Here, discussion switched from agriculturally-relevant pathogens, to fundamental genetic work on a model fungal pathogen of humans, *Aspergillus fumigatus*. *A. fumigatus* is an opportunistic human pathogen causing allergic reactions or systemic infections such as invasive pulmonary aspergillosis, especially in immune-compromised patients. In an example of principles that extend beyond plant pathogens to virulence mechanisms in general, Goldman and coworkers indicated that *A. fumigatus* mitogen-activated protein kinases (MAPKs) are involved in maintaining the normal morphology of the cell wall and providing resistance against cell wall-damaging agents (de Bruder Nascimento et al. 2016). Upon cell wall stress, cell wall-related sugars are synthesized from carbohydrate storage compounds. This process is dependent on cAMP-dependent protein kinase A (PKA) activity and regulated by the high-osmolarity glycerol response (HOG) MAPKs SakA and MpkC (de Assis et al. 2018). These protein kinases are necessary for normal accumulation/degradation of trehalose and glycogen, and the lack of these genes reduces glucose uptake and glycogen synthesis. Alterations in glycogen synthesis were observed for the *sakA* and *mpkC* deletion mutants, which also displayed alterations in carbohydrate exposure on the cell wall (de Assis et al. 2018). Carbohydrate mobilization is controlled by SakA interaction with PkaC1 and PkaR, suggesting a putative mechanism whereby the PkaR regulatory subunit leaves the complex and releases the SakA-PkaC1 complex for activation of enzymes involved in carbohydrate mobilization (de Assis et al. 2018). This suggest that reduced mobilization of monosaccharides for fungal cell wall biosynthesis during cell wall damage and the osmotic stress response can causes defects in the structure of the fungal cell wall, making these pathways possible targets for new antifungal strategies. Elucidation of the cooperation between the HOG and PKA pathways in the mobilization of

carbohydrates for fungal cell wall biosynthesis was reported. The reduced mobilization of simple sugars was suggested to cause defects in the structure of the fungal cell wall. In summary, it was proposed that SakA is important for PKA activity, therefore regulating the availability and mobilization of monosaccharides for fungal cell wall biosynthesis during cell wall damage and the osmotic stress response.

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

The Thon group provided a perspective of the fungal response to host carbohydrate and protein content, presenting an example from studies of the 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 Todd lab reported on the molecular genetics of branched chain amino acid (BCAA) leucine biosynthesis 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 each of the six BAT genes failed to confer 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 in 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.

Nutritional factors and host metabolism

Investigating the *Colletotrichum higginsianum* - *Arabidopsis* 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.

Voll and Gebauer also 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 to uptake organic building blocks from the host (Sutton et al. 1999; Fotopoulos et al. 2003), the finding by Chen et al. (Chen et al. 2010) led to the hypothesis that At*SWEET12* might accumulate in the extrahaustorial membrane to provide sucrose to the encased fungal haustoria. However, localization of the reporter gene fusions in powdery mildew infected leaves (Fig. 4 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, suggesting that the transporters are engaged in modulating phloem loading in infected leaves.

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 achieved using model fungi such as *Neurospora crassa* and *Aspergillus nidulans*.

Effectors

Secreted effectors are a key part of fungal-plant interactions, along with metabolic factors. Significant 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).

Conclusions

Of the questions asked at the workshop, several stand out:

How does the metabolic regulation by fungi occur during the transition 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 versus 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.

Nutritional factors and host signals may control the formation of macro- and microconidia of foliar leaf pathogens. Questions remain about whether and how nutritional factors might integrate with other signaling pathways during infection. For example, in *M. oryzae*, how does host cell viability signal the activation of effector deployment during biotrophy? Furthermore, colonization was widely discussed at the workshop; one intriguing aspect centered on how invasive hyphae that rapidly colonize living host cells and secrete effector molecules to suppress host immunity, cross into neighbouring cells at plasmodesmata. Through this structure, a range of nutritional molecular cargo is transferred, creating a sugar gradient. Do these nutrient gradients contribute to the transverse penetration of the constricted hyphae through plasmodesmata and contribute to fungal colonization?

Nutrient metabolic differences between oomycete species such as differential carbon metabolism or those observed in fruit pathogens that contribute to pH modulation, may also affect the variation in gene expression

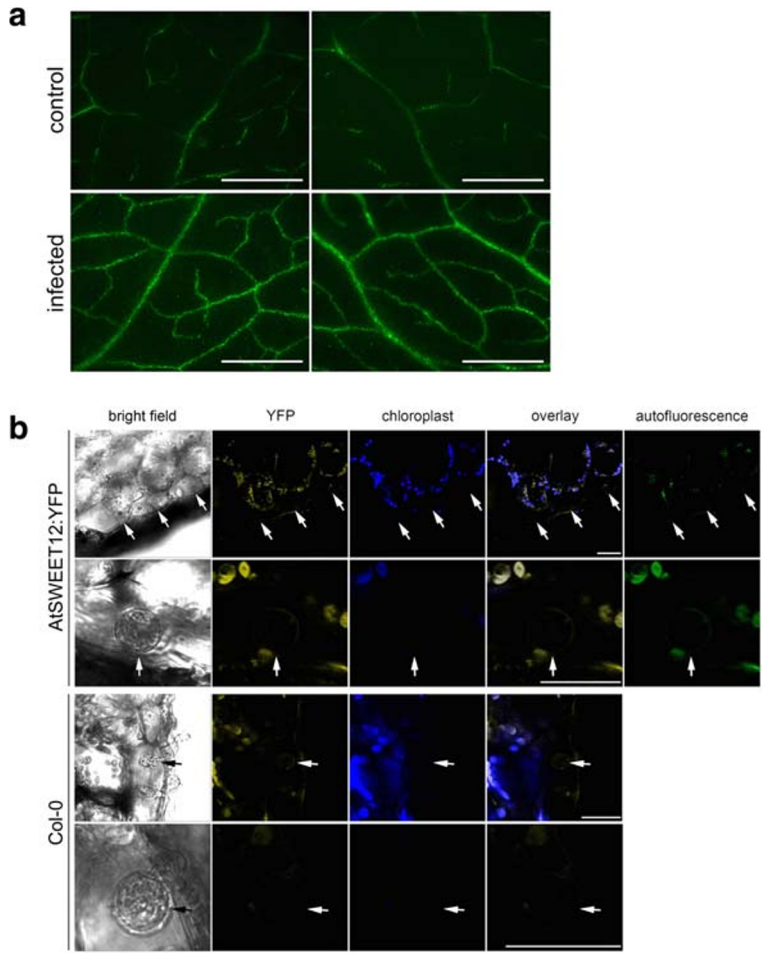

Fig. 4 Induction and localization of *pAtSWEET12*:*At*SWEET12-YFP upon *E. cruciferarum* infection in the vasculature, **a**) Fluorographs at binocular resolution were taken from control (top row) and *E. cruciferarum* infected plants (bottom row) 5 days after inoculation with 68 conidia mm^{-2} at a stage in which infected leaves were covered with a dense epiphytic mycelium. The scale bar indicates 1 mm. Brightness of all fluorographs has been digitally enhanced by the same factor. **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. Brightness of all fluorographs has been digitally enhanced by the same factor.

and enzyme catalytic behaviour. This may suggest the importance of nutrient availability. Nutrients may also affect the differential ROS- response of hemi-biotrophs and necrotrophs. Environmental conditions may, be critical for differential cell viability and/or nutritional availability. On the other hand, the reduced mobilization of simple sugars in *Aspergilli* 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 signaling 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, as shown for 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. As summarized above, these pH signals contribute to the activation, at the level of gene expression, of pathogenicity factors. 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. 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, 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. Furthermore, mechanisms of resistance and accumulation of the phytoalexin camalexin were reduced in starch-free mutants at late interaction stages.

The aim of the workshop 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 or in ripening fruits, for example, clearly leads to dynamic nutritional changes which have been addressed in depth in plant pathology. The challenge is now to understand how these programs limit or promote development of pathogens, as related to plant disease. The activation of pathogenicity factors, (including elicitors, enzyme secretion, modification of local pH and ROS levels, to mention a few topics covered at the workshop) affects colonization and development of lesions. To return to the question of senescence and ripening, the differential virulence levels of pathogens observed in fruit and vegetable tissue during ripening, resulting in increased nutritional availability and senescence, give rise to several questions about the importance of these changes for agricultural yield and food quality. Together, these findings indicate that understanding the genetic pathways and the consequent metabolic processes in pathogenic fungi that modulate their environment is important for developing effective disease-prevention strategies (Fernandes et al. 2017; Vylkova 2017). This shows 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.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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