Molecular aspects in pathogen-fruit interactions: Virulence and Resistance

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ABSTRACT

Fruit losses during postharvest storage and handling due to pathogen infections are one of the major problems in the global food chain supply. The application of chemical fungicides to control diseases is currently limited by legislation in some countries and also raises concerns about food and environmental safety. Exploring molecular aspects of pathogen-fruit interactions therefore has biological and economic significance as a means to help develop rational alternatives for disease control. In this review we present the current knowledge of molecular aspects in pathogen-fruit interactions, addressing the following topics: the application of new "omics" technologies for studying these interactions; the molecular mechanisms of fungal pathogen attack; the regulation of virulence by exogenous factors; and, finally, fruit defense mechanisms.

Contents

1. Introduction

Postharvest diseases caused by fungal pathogens lead to huge economic losses worldwide every year. Currently, the use of synthetic fungicides constitutes the main means to control these diseases. However, the development of resistance in fungal pathogens to fungicides and the growing public concern over the health and environmental risks

associated with high levels of pesticides in fruits have resulted in significant interest in developing alternative non-chemical methods of disease control (Mari et al., 2014). Furthermore, despite the application of fungicides and the increased use of new alternative strategies, fruits continue to be exposed to high infection pressure during production and commercial processing. In order to increase the arsenal of tools available to fight fruit decay we need to develop new approaches. In this context, the study of fruit-host interactions has gained increasing interest.

In the present review we have followed the nomenclature adopted by the American Phytopathological Society to define the terms pathogenicity and virulence (Sacristán and García-Arenal, 2008). Pathogenicity denotes the ability of a pathogen to cause disease on a particular host (a qualitative trait), whereas virulence is the degree of damage caused to the host (a quantitative trait). Virulence factors may be defined as those pathogen components that are non-essential for *in vitro* growth in a culture medium but contribute to disease. When one of these factors is required for pathogenicity it can be considered a pathogenicity factor. As virulence factors are important for infection, preventing pathogens from producing them constitutes an interesting alternative strategy for disease control. This strategy has been termed "antivirulence therapy" (Cegelski et al., 2008). As this therapy aims to disarm the pathogen rather than kill it or halt its growth, it is presumed that antivirulence compounds will generate much less selection pressure than traditional antibiotics in the pathogen to regain resistance, a problem faced with commonly used fungicides. However, before any antivirulence therapy can be developed there is a need to identify the virulence factors. In a broad sense, these factors can be as diverse as plant cell wall degrading enzymes (CWDEs), effectors (molecules that modify the physiology of the host in order to allow pathogen infection), or mechanisms that permit the rapid

adaptation of the pathogen to the host environment. The regulatory systems that govern all these processes can also be considered as virulence factors. Most of the research on antivirulence factors has been conducted with human pathogenic bacteria, although research on preventing fungal infections by *Candida albicans* and *Aspergillus fumigatus* has already been actively explored (Cui et al., 2015).

It is well documented that in many instances the differences in the outcome of a host-pathogen interaction (whether it is a compatible interaction, i.e., where disease occurs, or an incompatible/non-host interaction, i.e., where disease does not occur) depend on a rapid and efficient deployment of defense responses (Ferreira et al., 2006). These defenses are complex and constitute a multilevel series of structural and biochemical barriers that are either constitutive, preformed, and/or inducible. A first line of defense is common against all potential pathogens and is triggered by pathogen-associated molecular patterns (PAMPs). When a successful pathogen is able to escape this defense, then a second defense line is deployed by the host, the so called effector-triggered immunity. Plants respond to pathogen attack even in compatible interactions, although this response is insufficient to avoid infection progress. One of the earliest responses detected in many incompatible interactions (i.e., where the plant is resistant to the pathogen) is the activation of an oxidative burst characterized by an increase in the levels of reactive oxygen species (ROS) (Pitzschke et al., 2006). The oxidative burst precedes the synthesis of antimicrobial compounds like phytoalexins. This burst leads up to alterations in the synthesis of cell-wall structural proteins and the transcriptional activation of specific genes leading to the synthesis of pathogenesis-related (PR) proteins such as chitinases, β-1,3-glucanases and peroxidases (POD). Although these defense factors in host tissues

have been extensively studied, the mechanisms that regulate these responses have not been elucidated.

The application of molecular genetics techniques has significantly changed the study of plant-pathogen interactions, enhancing scientists' ability to test hypotheses and thus provide new information on the biochemical mechanisms underlying these interactions, i.e., pathogen virulence and host resistance (An et al., 2016a; Frías et al., 2011; Schouten et al., 2008). Cloning and the characterization of crucial genes in several fungal pathogens that are related to virulence/pathogenicity are likely to lead to a deeper understanding of the molecular mechanisms underlying fruit disease susceptibility/resistance. The above mentioned facts justify the need to increase our knowledge of the mechanisms of pathogen virulence and host fruit defense. This knowledge may lead to the rational design of new and safer control strategies.

2. New technologies for studying host-pathogen interactions in postharvest fruit systems

During the last ten years we have witnessed an "omics" revolution that has involved all fields of biological sciences. This change is reflected in the way researchers now go about the study of biological processes, from a single gene/enzyme level to a holistic approach through which the researchers try to examine problems from a global point of view. Sequencing technology and its applications have expanded from the sequencing of individual genes to the assembly of complete genomes, from the analysis of the expression of a few genes to global transcriptomic analyses, and so on. However, the implementation of these new technologies in the field of postharvest pathology is lagging behind their uses in other closely related fields. "Omics" technologies are being used to

investigate many fruit crops from other points of view, and the information gathered from those studies can be used in postharvest pathology. Fruit crop genomes that have been sequenced include grape (Jaillon et al., 2007); apple (Velasco et al., 2010); banana (D'Hont et al., 2012); citrus (Xu et al., 2013); peach (Verde et al., 2013); and pear (Chagné et al., 2014). The genomes of many fungal plant pathogens have also been sequenced in recent years. The fungal sequencing genome projects contained in MycoCosm (Grigoriev et al., 2013) (http://genome.jgi.doe.gov/pages/fungi-1000-projects.jsf) provide a picture of the rapid progress being made in this field. The genomes of several postharvest pathogens are now available, including *Botrytis cinere*a (Amselem et al., 2011) and several different species of *Alternaria* (Dang et al., 2015) and *Colletotrichum* (Gan et al., 2013). The genomes of the following specific postharvest fruit pathogens from the genus *Penicillium* have also been recently sequenced: *P. digitatum* (Marcet-Houben et al., 2012; Sun et al., 2011); *P. griseofulvum* (Banani et al., 2016); and *P. expansum* and *P. italicum* (Ballester et al., 2015; Li et al., 2015). There are also ongoing genome sequencing projects for other relevant postharvest fungi, such as *Monilinia fructicola* and *M. laxa*.

The genetic information gathered in these projects provides a foundation for an in-depth analysis of the virulence factors of these important postharvest pathogens. Data are available on the whole set of putative genes present in these pathogens and on how many of these genes are secreted, which ones are candidate effectors and the arsenal of carbohydrate-active enzymes (CAZymes). There is also data on the array of proteases that each fungus can produce. In order to decipher which genes are relevant to pathogenesis and virulence, however, more studies are needed. Genes involved in pathogenicity and virulence are actively expressed during the colonization of the host. A

transcriptomic analysis of the pathogen-fruit interaction will thus reveal these genes, as well as other genes that are necessary for the normal growth of the fungus and are not necessarily related to pathogenicity/virulence. One advantage of directly analyzing the host/pathogen interaction is that information on host defense responses deployed in response to the fungal attack can also be obtained. The first approach used to analyze the fruit-pathogen interactome at the transcription level was based on sequencing ESTs (expressed sequence tags) (González-Candelas et al., 2010a) and on the identification of differentially expressed genes (Sánchez-Torres and González-Candelas, 2003). Other approaches that allow us to identify genes from either the fruit or the pathogen that are overexpressed during their interaction are based on the analysis of cDNA libraries using suppression subtractive hybridization (SSH) (Casado-Díaz et al., 2006; González-Candelas et al., 2010b; López-Pérez et al., 2015) or microarray hybridization (Guidarelli et al., 2011; Vilanova et al., 2014b). One advantage of the SSH approach is that it does not require previous knowledge of either the host or the pathogen, whereas a microarray can only be prepared once a large set of cDNAs from either interacting partners has been isolated. Recent advances in high-throughput sequencing technologies and decreasing costs have enabled small labs to conduct complex transcriptomic analyses using a whole transcriptome shot-gun sequencing approach (RNA-Seq). RNA-Seq, with its capability of producing millions of sequences from complex RNA samples, is displacing microarray analysis as the preferred tool for conducting global transcriptomic studies. Researchers have only recently begun to apply RNA-Seq in fruit-pathogen studies. Blanco-Ulate et al. (2014) investigated the expression of CAZymes-encoding genes in *B. cinerea* during its interaction with lettuce leaves, tomato fruit and grape berries, highlighting host-specific commonalities and differences. The interaction between apple fruit and *P. expansum* has

also been recently studied using RNA-Seq to analyze fungal genes expressed during colonization at 24, 48 and 72 h post-inoculation (hpi) (Ballester et al., 2015). Several genes classically related to virulence were induced, such as proteases, CWDEs and oxidoreductases. These types of enzymes are typically encoded by genes that are part of large gene families. By sequencing the global RNA population, one can determine which genes are actually induced. It was thus found that an aspergillopepsin-encoding gene showed the highest expression level at just 24 hpi, and that different pectinase-encoding genes exhibited different patterns of regulation during the course of infection. RNA-Seq also made it possible to discover genes that may represent putative virulence/pathogenicity factors. Previous studies had shown that *P. expansum* produces two glucose oxidases (GOX1 and GOX2), but only one of them, GOX2, has been implicated in virulence (Barad et al., 2012). In the RNA-Seq study a new *GOX3*-encoding gene was found that exhibited an earlier and much higher induction level than *GOX2*.

Although knowing which genes are expressed is highly useful, it is possible to directly analyze the proteins being synthesized during a fruit-pathogen interaction using a proteomic approach. This approach bypasses the potential of a post-transcriptional regulation level and provides direct information on the final players, the proteins.

Several studies, mainly on *B. cinerea* and *P. expansum*, have analyzed proteins synthesized by fungal postharvest pathogens under conditions that could affect pathogenesis, such as during conidia germination or exposure of the fungus to different pH values (Li et al., 2010; 2012), and under conditions that simulate the presence of the host (for a recent review see González-Fernández et al., 2015). Similarly, some proteomic studies have also been conducted in fruits to analyze their response to elicitors of induced resistance (Chan et al., 2007; Chan et al., 2008); wounding (Buron-Moles et al., 2014);

or to *Penicillium* spp. (Buron-Moles et al., 2015b). There has been only one study, however, that has analyzed the interacting proteome of both the fruit and pathogen simultaneously. This study conducted by Shah et al. (2012) profiled the proteome of ripening tomato fruits during its interaction with *B. cinerea*.

Another approach for studying the interaction between fruits and pathogens is to analyze the physiological and biochemical changes that occur using metabolomics. Most of the metabolomics studies conducted so far in fruit- pathogen interactions have been focused on the defense mechanisms of the fruit, in particular on the analysis of phenolic compounds, because these compounds have been classically associated with increased resistance levels in fruits.

The metabolite profiling of citrus fruit inoculated with *P. digitatum* showed an induction of different flavanones, flavones, polymethoxylated flavones and scoparone (Ballester et al., 2013b). Some of these metabolites are also induced in fruits in which increased resistance to pathogens has been triggered by an elicitor (Ballester et al., 2013a), although other phenolics found in elicited fruit were not detected in response to *P. digitatum* infection. The role of polymethoxylated flavones in disease resistance has also been revealed in the metabolomic analysis of a spontaneous yellow citrus mutant that is more resistant to green mold infection (Luo et al., 2015). In a recent study involving 24 apple varieties, correlation analysis revealed that phenols like flavonols and procyanidins B2 in the peel fraction of the inoculated fruit have a significant impact on blue mold resistance (Ahmadi-Afzadi et al., 2015). The comparison of tomato fruits that accumulate different levels of flavonoids and show different ripening behavior has also shed light on the role that specific flavonoids with high superoxide scavenging activities play in the higher resistance of some tomato lines to *B. cinerea* infection (Zhang et al., 2015).

Whatever the source of information regarding the possible involvement of a gene/protein in a host-pathogen interaction, either as a virulence factor or as a resistance mechanism, there is a need to validate such involvement. This functional analysis is normally achieved by manipulating the expression level of the corresponding gene. Obtaining gene knockout mutants is considered the most direct way to confirm the involvement of a gene in a biological process. In some instances, when deletion of a gene is not feasible, the analysis of mutant lines where the gene has either been downregulated or overexpressed can give a solid clue as to its function. For some postharvest fungal pathogens, such as *B. cinerea*, there are numerous articles describing the characterization of gene knockout mutants. There are many cases in which the gene deletion mutant shows no difference in virulence, or just a small decrease, with respect to the wild strain. One possible reason is the existence of multigenic families, such as those of CWDEs or protease encoding genes. When a gene belonging to a multigenic family is deleted, another member of the family can compensate for the missing one. One extreme example is the proteinase gene family of *B. cinerea*, where single and double gene knockout mutants were not affected in virulence (ten Have et al., 2010). However, the loss of a single polygalacturonase-encoding gene in an *Alternaria alternata* isolate causing citrus black spot reduced the virulence towards citrus fruit, whereas the loss of the orthologous genes in another *A. alternata* isolate causing citrus brown spot had no effect on virulence (Isshiki et al., 2001). The Host-Pathogen interaction database (PHI-base; http://www.phi-base.org) (Urban et al., 2015) contains more detailed information on the genes from different fungal pathogens that have been analyzed in the context of host-pathogen interactions.

The same functional analysis approach is useful for analyzing the putative role of plant genes in the defense against pathogens. Limonene is a terpene that accounts for up to 97%

of the volatile emission of sweet orange and is an inductor of spore germination in *P. digitatum*, but it does not induce germination in the non-pathogen *P. expansum* (Droby et al., 2008). Downregulation of the citrus limonene synthase by antisense RNA led to transgenic citrus fruit with reduced levels of limonene that were less susceptible to *P. digitatum* infection (Rodríguez et al., 2011).

3. Molecular mechanisms of fungal pathogenicity

3.1. Extracellular enzyme determinants: Phytopathogenic fungi invade and colonize their host cell to obtain nutrients. Necrotrophic fungi kill their host cell before and during tissue invasion without a specific invasion of cells. These latter fungi thrive on dead organic material that is no longer a cell. This complex process requires the help of a large number of extracellular hydrolytic enzymes. Previous studies demonstrated that fungal pathogens secrete a large set of proteins into the extracellular environment (Kim et al., 2008). A number of secreted proteins are crucial for the virulence of pathogens, particularly the necrotrophic ones. Growing evidence has demonstrated that the extracellular CWDEs of pathogens, including endopolygalacturonases (Kars et al., 2005; ten Have et al., 1998), pectin methylesterases (Valette-Collet et al., 2003), endo-1,4-β-glucanases (Eshel et al., 2000) and xylanases (Brito et al., 2006), are necessary for successful establishment and proliferation (lesion extension) in plant tissues. There are at least six endopolygalacturonase genes in *Botrytis cinerea* (Wubben et al., 1999). The deletion of two endopolygalacturonase genes, separately, resulted in a pronounced reduction of virulence (Kars et al., 2005; ten Have et al., 1998). Furthermore, demethylation by pectin methylesterase presumably preceded and facilitated the action of endopolygalacturonases (Valette-Collet et al., 2003). Besides CWDEs, extracellular

proteins with other functions also contribute to the virulence of fungal pathogens. Rolke (2004) showed that the secreted superoxide dismutase played an important role during cuticle penetration by the appressorium of *B. cinerea*. The knockout of the superoxide dismutase encoding the gene *Bcsod1* in *B. cinerea* led to significantly reduced virulence. An oxidative burst has been observed during cuticle penetration, and BcSOD1 may be involved in this process. In addition, a few secreted proteins have the ability to induce a hypersensitive response in the host, including, among others, Nep1-like proteins (NLP) (Schouten et al., 2008); cerato-platanin family proteins (Frías et al., 2011); and xylanases (Noda et al., 2010). The NLPs were first described in *Fusarium oxysporum* and constitute a growing family of known secreted proteins produced by fungi, oomycetes and bacteria (Gijzen and Nürnberger, 2006).

Extracellular proteins have also been found to be involved in the molecular dialogue associated with host-pathogen interactions (Esquerré-Tugayé et al., 2000), suggesting that a precise regulation of these proteins may occur during pathogenesis. In fungal pathogens, RabGTPases act as a vital regulator of the secretion process of extracellular proteins. CLPT1, a putative SEC4 homologue in the filamentous fungus *Colletotrichum lindemuthianum*, was shown to be essential for the transport of the secretory vesicles and virulence (Dumas et al., 2001; Siriputthaiwan et al., 2005). The Rab gene *Bcsas1* of *B. cinerea*, which is the homologue of Rab8 in mammalians, was found to be crucial for growth, development, protein secretion and virulence (Zhang et al., 2014b). The knockout of *Bcsas1* inhibited the secretion of virulence factors, such as polygalacturonase and xylanase, in the extracellular environment, and significantly decreased the virulence of *B. cinerea*. These findings suggest that extracellular proteins and the regulation of the secretion process are crucial for pathogenesis.

3.2. Reactive oxygen species and NADPH oxidase complexes: Many investigations during recent decades have suggested that ROS play a major role in plant-microbe interactions. ROS are normal by-products of many metabolic reactions and include superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). ROS can act as potent oxidants or signal molecules during host-pathogen interactions, thus playing a dual role. On the one hand, plants react to microbe infection with an oxidative burst, which is necessary for further defense reactions. On the other hand, it has been shown that some pathogenic fungi can also produce ROS, which play an important role in the differentiation process and virulence. During the infection process of *B. cinerea*, $O_2^{\cdot -}$ accumulates in the hyphal tips and H_2O_2 is generated in and around the penetrated cell (Tenberge et al., 2002). Castoria et al. (2003) showed that the calibrated addition of exogenous superoxide dismutase and catalase in apple wounds strongly reduced both $O_2^{\cdot -}$ and H_2O_2 generation and the level of infection by *B. cinerea*. However, the role of ROS in fruit-pathogen interactions seems to be complex and could be different depending on the pathogen. A higher induction of H_2O_2 in less mature apples, for example, has been associated with less susceptibility to *P. expansum* infection (Torres et al., 2003). In fact, effective biocontrol yeasts not only induce ROS production (Macarisin et al., 2010) but are also more tolerant to ROS (Castoria et al., 2003). In eukaryotic organisms, ROS are generally produced in mitochondria or through specialized enzymes. Mitochondria are considered to be the main source and target of intracellular ROS (Tian et al., 2013). The most important enzymatic ROS-generating systems are the NADPH oxidase (Nox) complexes. The Nox complexes use NADPH as an electron donor and transport electrons through the membrane, either the plasmatic membrane or an internal vesicle membrane,

to reduce oxygen to superoxide (Lambeth, 2004). In fungi, the Nox complexes are associated with a wide range of functions, such as sexual differentiation and pathogenicity. In *B. cinerea*, two homologs of the human catalytical subunit Nox2 have been identified and designated as NoxA and NoxB, respectively. Both NoxA and NoxB are required for the formation of sclerotia (Segmüller et al., 2008). The functions of the Nox complexes in pathogenesis have been intensively studied in recent years. These complexes are associated with the differentiation of infection structures such as appressoria and penetration hyphae. Both NoxA and NoxB have a great impact on pathogenicity (Segmüller et al., 2008). NoxB mutants showed a retarded formation of primary lesions. NoxA mutants, on the other hand, were able to penetrate host tissue in the same way as the wild type but were much slower in colonizing the host tissues. The double knockout mutants of NoxA and NoxB showed an additive effect: they were almost nonpathogenic. Both proteins are regulated by the regulatory subunit NoxR, the homolog of the mammalian p67phox, and a small GTPase Rac (Segmüller et al., 2008; Takemoto et al., 2007). The small GTPase Rac is activated by the GDP/GTP exchange factor (GEF) and binds to NoxR. Homologs of Rac have been identified in several filamentous fungi, but have not yet been found in *B. cinerea* (Takemoto et al., 2007). An et al. (2015) reported that a small GTPAse, Rho3, a potential subunit of Nox complexes, was involved in the accumulation of ROS in hyphae tips and affected the sporulation, appressorium formation and virulence of *B. cinerea*. Interactions between Rho3 and other subunits of the Nox complexes are unknown. Recently, another component of Nox complexes, NoxD, which has high homology to the endoplasmic reticulum (ER) protein Pro41 from *Sordaria macrospora*, has been identified (Siegmund et al., 2015). NoxD can interact with NoxA and shows similar functions to NoxA in differentiation and pathogenicity.

These data provide evidence that certain functions of the NoxA complexes of *B. cinerea* are linked to the ER (Siegmund et al., 2015).

As important second messengers, ROS mediate a signaling pathway in fungi (Heller and Tudzynski, 2011). The oxidative stress initiates the phosphorylation of the MAP kinase Sak, a key component of the signal transduction pathway. Sak is associated with vegetative growth, sporulation and pathogenic development (Segmüller et al., 2007). In addition, some transcription factors (TFs) also participate in the ROS signaling pathway, such as Arf1 and Bap1 (Williamson *et al.*, 2007; Temme *et al.*, 2012). How ROS are transported is an interesting question. Previous studies suggest that the transport of H_2O_2 in plants and mammals is mediated by aquaporins (AQPs) (Bienert et al., 2007). AQPs are water-channel proteins that generally mediate the selective and rapid flux of water across biological membranes. Recently, An et al. (2016) identified eight *AQP* genes in the genome of *B. cinerea*. Among these genes, *AQP8* was involved in ROS production, distribution and transport across membranes. The deletion of *AQP8* completely inhibited the formation of conidia and infection structures and markedly reduced the virulence of *B. cinerea* in tomato leaves and fruit. In addition, the deletion of *AQP8* significantly reduced the expression of the *noxR* gene, suggesting that AQP8 may affect the function of the Nox complexes by influencing the expression of the *noxR* gene at a transcriptional level. Moreover, the knockout of both *AQP8* and *noxR* changed the distribution of mitochondria. These results indicate that there is a complicated ROS regulatory network in fungi (Fig. 1).

3.3. Effectors: The fungal pathogens are usually distinguished as either biotrophic (acquiring nutrition directly from living plant cells); hemibiotrophic (with an early biotrophic phase followed by a switch to necrotrophy); or necrotrophic (killing plant cells

and feeding on the dead cells). The traditional theory holds that biotrophic pathogens have an intimate interaction with their hosts. Many of these pathogens can deliver effectors into host cells to suppress the host immune response. Kleemann et al. (2012) revealed that the hemibiotrophic pathogen *Colletorichum higginsianum* also contains a large inventory of candidate effectors, and most effector genes are host-induced and expressed in consecutive waves associated with the transition from biotrophic to necrotrophic growth. Most postharvest pathogens, such as *B. cinerea*, *P. expansum* and *A. alternata*, are classified as necrotrophic fungi. These fungi have long been considered to be unsophisticated pathogens that do not have effectors and kill host cells with the use of CWDEs or toxins. However, some recent studies have changed this simplistic perception. Zhang et al. (2014) found that during the infection process the endopolygalacturonases not only degraded the cell wall of host cells, but also could be recognized as microbe-associated molecular patterns by the *Arabidopsis* receptor-like protein RBPG1, thereby influencing the resistance of the host. Recent discoveries of fungal small RNAs, which can be delivered into host cells to suppress plant immunity, added small RNAs to the list of pathogen effectors. Weiberg et al. (2013) found that some *B. cinerea* small RNAs could be injected into the host cells and act as effectors. These small RNAs hijack the host RNA interference (RNAi) machinery by binding to *Arabidopsis* Argonaute 1 (AGO1) and selectively silencing host immunity genes. The finding of effectors in the so-called necrotrophic fungi provides new insights into the interaction between postharvest pathogens and hosts.

4. **Regulation of virulence by exogenous factors**

Fungi, as multicellular microorganisms, are sensitive to exogenous factors. It is well known that environmental conditions like nutrient availability, temperature, and relative humidity have a significant effect on the growth, reproduction and pathogenicity of plant pathogens. Recently, the roles of other environmental conditions, including ambient pH and light, have attracted more and more researchers' attention. In addition, exogenous substances, which are applied to control postharvest diseases, have also been shown to have significant effects on pathogenicity.

4.1. Ambient pH: Ambient pH is an important environmental factor that has significant effects on the physiology and pathogenicity of fungi. Li et al. (2010) found that ambient pH might affect spore germination in *P. expansum* by changing intracellular pH and ATP levels and the synthesis and folding of proteins. Manteau et al. (2003) found that ambient pH could influence the activities of extracellular proteases, polygalacturonases and laccases in *B. cinerea*. Moreover, *Bcpg*1-6, genes encoding endopolygalacturonases, responded differentially to different ambient pH values, both *in vitro* and *in vivo* (ten Have et al., 2001; Wubben et al., 2000). These secreted proteins are considered as potential virulence factors during the infection process. Li et al. (2012) analyzed the changes in the whole secreted proteins (secretome) of *B. cinerea* under different pH values using 2-DE. The values pH 4 and 6 were selected to mimic the pH values of different host tissue: pH 4 represents the pH value of fruit, and pH 6 represents the pH value of stems, flowers and leaves. A total of 47 differential protein spots (corresponding to 21 unique proteins) were identified, and most of these proteins were CWDEs or proteases. Interestingly, these researchers found that more proteins related to proteolysis were induced at pH 4, whereas more CWDEs were induced at pH 6. These findings suggest that inducing the secretion of proteases, rather than CWDEs, is more important

when *B. cinerea* infects ripe fruits, which have lower tissue pH and weakened cell walls. In contrast, CWDEs are more urgently required during the infection of leaves and stems, which have higher tissue pH and stiffer cell walls. The smart switch of secretome responding to different ambient pH values may contribute to a broad range of hosts in *B. cinerea*.

Fungi possess a complex system to sense and respond to changes in ambient pH. This system has been well characterized in *Aspergillus nidulans*, in which the following seven genes have been identified: *pac*C, *pal*A, *pal*B, *pal*C, *pal*F, *pal*H and *pal*I (Peñalva et al., 2008). In the system, *pacC* encodes a pH-dependent global transcription factor and plays a critical role in regulating gene expression. The *pacC* gene has also been found in several other postharvest fungal pathogens and contributes to the full virulence of these pathogens, including *Colletotrichum gloeosporioides* (Alkan et al., 2013); *P. expansum* (Barad et al., 2014); and *P. digitatum* (Zhang et al., 2013). In *P. expansum*, ambient pH and *pacC* are also involved in the secondary metabolism and have been shown to affect the production of the mycotoxin patulin (Barad et al., 2015; Zong et al., 2015). Although there are conflicting reports on the possible role of this mycotoxin in pathogenesis (Ballester et al., 2015; Barad et. al., 2014; Li et al., 2015; Sanzani et. al., I2012), a recent study by Snini et al. (2015) indicates that patulin acts as a cultivar-dependent aggressiveness factor for *P. expansum.* In *B. cinerea*, the level of gene expression of *BcpacC* was found to be markedly higher at pH 6 than that at pH 4, indicating that *pacC* is also involved in the response of *B. cinerea* to ambient pH (Li et al., 2012).

Fungal pathogens not only passively sense and adapt to ambient pH, but also can positively change it to facilitate their infection of hosts by secreting alkaline or acid substances. Prusky and Yakoby (2003) defined fungi that produce an alkaline

environment as ‘alkaline fungi’, and those that produce an acidic environment as ‘acidic fungi’. *C. gloeosporioides* and *A. alternata*, as typical alkaline fungi, can secrete ammonia to alkalinize the ambient pH of infection sites and induce the expression of genes encoding virulence factors (Eshel et al., 2002; Kramer-Haimovich et al., 2006). In contrast, gluconic acid and oxalic acid produced by acidic fungi like *P. expansum* and *B. cinerea* can acidify the ambient pH, which results in higher CWDE activity at the lower pH level (Manteau et al., 2003; Prusky et al., 2004).

4.2. Light: Like ambient pH, light is also an important environmental factor capable of affecting the development, reproduction and metabolism in filamentous fungi. In fungi, the light signaling pathway has been best studied in *Neurospora crassa*. A similar mechanism has also been described in *A. alternata* by Pruss et al. (2014). The pathway is mediated via the WHITE COLLAR transcription factors WC-1 and WC-2 that form a complex (WCC) in response to blue light, leading to the activation of gene expression (Chen et al., 2010). A similar system has also been identified in *B. cinerea*, although the light-associated machinery seems more complicated in *B. cinerea* than in *N. crassa*. Canessa et al. (2013) found that not all wild isolates of *B. cinerea* exhibited phenotypic responses to light because of a high level of genetic variation in the species. In strain B05.10, light was a key environmental signal influencing the mode of (asexual) reproduction, growth and pigment accumulation, and WCC (BcWCL1 and BcWCL2) was an integral part of the light-associated machinery by mediating transcriptional responses to white light. Furthermore, the WCC is required to respond to oxidative stress and to achieve full virulence. Besides *bcwcl1*and *bcwcl2, B. cinerea* possesses another key gene in the light-signaling pathway, *bcfrq1*, which encodes a protein with 31.3% identity to its *Neurospora* homolog (Hevia et al., 2015). In this study, the Δbcfrq1 mutant

presented decreased macroconidiation, enhanced sclerotia formation and impaired virulence on leaves of *Arabidopsis thaliana* Col-0.

Recently, Schumacher et al. (2014) identified a novel light-induced GATA transcription factor BcLTF1 (*B. cinerea* light-responsive transcription factor 1) by a random mutagenesis approach. BcLTF1 regulated not only the light-dependent differentiation, but also the equilibrium between the production and scavenging of ROS. Compared with the wild type, the bean leaves of the *bcltf1* deletion mutant showed greater ROS accumulation and smaller lesion diameters 3 days after inoculation. Microarray analyses revealed that 293 light-responsive genes were modulated by BcLTF1. To date, however, the relationship between BcLTF1 and the WCC complex remains unknown.

4.3. Exogenous substances: As an alternative to fungicides, a number of exogenous substances, such as sodium bicarbonate, silicon, borates and essential oils, have been applied to control postharvest diseases with positive results (Calo et al., 2015; Fallanaj et al., 2016; Qin et al., 2007; Qin and Tian, 2005; Shi et al., 2012; Yao et al., 2004). On the one hand, these substances can activate the defense system and induce resistance against pathogens; on the other hand, they also show direct effects on the pathogens. Though most related studies have only reported the inhibitory effects of exogenous substances on pathogens, a few studies have carried out a deeper analysis of the antifungal mechanism of these substances. Qin et al. (2007) found that borates, essential plant micronutrients, show a significant inhibitory effect on the spore germination and germ tube elongation of *P. expansum*. Furthermore, 14 differentially expressed protein spots were identified under borate stress using 2-DE and ESI-Q-TOF-MS/MS. Among these proteins, catalase and glutathione S-transferase, the two antioxidant enzymes, were less abundant upon exposure to borate. This effect might contribute to enhanced levels of intracellular ROS

and protein carbonylation. Intracellular ROS accumulation under borate stress has also been observed in spores of *C. gloeosporioides*, and mitochondrial degradation might be involved in the mechanism of antifungal activity of borate (Shi et al., 2012).

5. **Unravelling fruit defense mechanisms**

Plants in general, and fruit in particular, are in constant contact with a large amount of microorganisms in the environment. Most of these microorganisms, however, are not able to produce infection (Ferreira et al., 2006). Recent studies have shown that either under specific conditions (Louw and Korsten, 2015; Louw and Korsten, 2014) or depending on the maturity stages of fruit (Vilanova et al., 2014a; Vilanova et al., 2012b), even some non-host pathogens are able to overcome fruit defenses and produce infection. These recent works have indicated that fruit maturity plays a main role in the development of postharvest diseases. Maturity is thus another major component in fruit resistance that should be considered in fruit-pathogen interaction studies.

Several species of postharvest pathogens, such as *Colletotrichum*, *Alternaria*, *Botrytis* and *Monilinia*, have been reported to remain quiescent in their hosts until the fruit ripens. In a recent study focused on tomato fruit defense responses against the fungus *C. gloeosporioides*, researchers showed that the expression of several fruit genes was induced even before appressoria penetration. Such genes included PAMP receptors and genes related with fatty acid biosynthesis, elongation, and the synthesis of cutin and waxes (Alkan et al., 2015). Moreover, the host defense reaction was further intensified during the quiescent stage by the activation of lignification, glycoalkaloid and phenylalanine pathways.

To unravel fruit responses against pathogen attack, it should be considered that fruits induce cellular defenses that prevent further colonization of the fruit tissue once the structural barriers have been breached by the pathogen. The structural or physical barriers "that pathogens have to break" are known as the passive (or constitutive) defense mechanisms of fruits to avoid pathogen ingress and/or progress. It has been reported that Trincadeira berries infected with *B. cinerea* accumulate saturated long-chain fatty acids, which are major constituents of grape waxes, and that this accumulation is accompanied by the up-regulation of several acyl-CoA synthetases and wax synthases (Agudelo-Romero et al., 2015). These studies show that the formation of barriers that are commonly recognized as constitutive can be induced within the framework of a host's active response to fungal attack. These findings warrant further attention and future research given the critical role host lipids and lipid metabolites play in the dynamics of fruit defense.

Other fruit defense mechanisms, described as active (or inducible) defense mechanisms, require host mechanisms to function. Knowledge of plant-fungal interaction indicates that a crucial role in host defense is played by a rapid and massive generation of ROS within the host cells, a process known as an oxidative burst (Torres et al., 2006). The oxidative burst is one of the earliest responses to pathogen attack and involves the production of ROS including the superoxide anion, the hydroxyl radical and H_2O_2. In spite of the important role of ROS in plant defense, there are few reports on the role that they might play in fruits. To date, the accumulation of H_2O_2 in response to wounding and/or pathogen attack has been demonstrated in tomatoes (Borden and Higgins, 2002); lemons (Macarisin et al., 2007); avocados (Castro-Mercado et al., 2009); plums (El-kereamy et al., 2009); blueberries (Miles et al., 2011); oranges (Buron-Moles et al., 2015a; Torres et al., 2011);

and apples (Buron-Moles et al., 2015a; Castoria et al., 2003; Su et al., 2011; Torres et al., 2003). The study by Castoria et al. (2003) also showed O_2^- production as a consequence of wounding. ROS metabolism is controlled by respiratory burst oxidase homologs (Rbos) and an array of other enzymes including superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (De Gara et al., 2003). In the orange-*P. digitatum* interaction, Ballester et al. (2006) found that the antioxidant activities of SOD, CAT, APX and glutathione reductase (GR) decreased at different rates with the advance of the fungus. In the area of the flavedo completely colonized by the fungus, however, the CAT and soluble POD activities increased. Moreover, all the enzyme activities were higher in non-infected areas of the flavedo than in the albedo, a fact that may be related to the greater resistance of the flavedo to infection by the pathogen. In apple-*Penicillium* interactions, Vilanova et al. (2014b) observed that Rbo gene expression was induced in apples inoculated with *P. digitatum* but not in apples inoculated with *P. expansum*. These researchers also noted a greater increase in the expression of genes encoding ROS-detoxifying enzymes, such as SOD, APX and POD, in apples inoculated with *P. expansum*. Buron-Moles et al. (2015b) also observed higher APX transcript abundance in apple in response to *P. digitatum* than in response to *P. expansum*. Additional proteomic data indicated that tomato infected by *R. nigricans* repressed the accumulation of catalase but induced the translation of catalase isozyme 2 (Pan et al., 2013). Further studies of gene expression should be done in order to understand these responses in depth.

The production of H_2O_2 in plants is also involved in lignification (Olson and Varner, 1993), and H_2O_2 is a signaling molecule mediating the induction of hypersensitive cell death and the expression of a wide array of defense-related genes in surrounding cells (Borden and Higgins, 2002; Grant and Loake, 2000). The lignification process may

contribute to fruit resistance in different ways: i) making the fruit more resistant to degradation by enzymes secreted by an invading pathogen; ii) constituting a barrier preventing free nutrient movement and therefore helping to starve the pathogen; and iii) slowing down the penetration process. Lignin deposition as a fruit response to abiotic stress (wound) or biotic stress (pathogen) has been demonstrated using histochemical analysis (Skene, 1981; Spotts et al., 1998; Vilanova et al., 2012a; Vilanova et al., 2012b) and by quantifying lignin (Su et al., 2011; Valentines et al., 2005; Vilanova et al., 2013; Vilanova et al., 2014a). The role of phenylalanine ammonia lyase (PAL), a key enzyme in the phenylpropanoid pathway, in regulating lignin accumulation (Lewis et al., 1999) and synthesizing phenols (González-Candelas et al., 2010a) has been described. A recent study on the orange-*P. digitatum* pathosystem detected the maximum expression of several phenylpropanoid-related genes at 48 h after inoculation (Ballester et al., 2013b). In addition, Vilanova et al. (2013) found that PAL1, caffeic acid O-methyl-transferase (COMT1) and POX1 gene expression were induced to a greater extent by *P. digitatum* than by *P. expansum*, although the maximum expression was observed at 24 h, which may be due to the inoculum concentration used. Similar results were obtained in apple responses at different maturity stages (Vilanova et al., 2014a), with the highest level of induction of several phenylpropanoid-related genes occurring at 48 h after *P. expansum* inoculation.

A further active defense mechanism is related to pathogenesis-related proteins (PR), which have diverse functions and most of which are induced by stress (biotic or abiotic) (van Loon et al., 2006b). In terms of the fruit defense response, diverse works have described both the enzyme activity and gene expression of PR-proteins. For example, an increase in chitinase and glucanase enzymatic activities was detected in tomato fruit as a

response to infection caused by *A. alternata* (Cota et al., 2007) and in grapes infected by *B. cinerea* (Salzman et al., 1998). In plums, researchers observed an increase in the transcript level of PR10 and in H_2O_2 production after *M. fructicola* infection (El-kereamy et al., 2009). Additionally, the gene expression of PR proteins [chitinase (CHI), PR10 and β-glucanase] was detected earlier in a susceptible variety after 24 h of *C. acutatum* inoculation (Miles et al., 2011). Furthermore, two PR genes (PR5 and PR10) were found to be over-expressed in *Fragaria chiloensis* and *Fragaria* x *ananassa* after *B. cinerea* infection and showed different expression profiles depending on the tissue analyzed (González et al., 2013). Vilanova et al. (2014b) showed that *P. expansum* inoculation in apples induced a greater number of PR proteins [two CHIs, endoglucanase (EGL), pathogenesis-related thaumatin (TAU), and the defensin-like protein (DEFL)], than inoculation with *P. digitatum*. Complementary proteomic analysis conducted in apple reported an increase in the abundance of the thaumatin-like protein 1-a (PR5), suggesting that it may be involved in the initial apple wound response (Buron-Moles et al., 2014). Additionally, three PR10 proteins were detected in proteomics studies on both wounded and inoculated apples with *P. expansum* and *P. digitatum* pathogens (Buron-Moles et al., 2015b).

Plant defenses are regulated by complex signaling pathways involving salicylic acid, jasmonic acid and ethylene. Specifically, ethylene has been implicated in biotic stress, both as a virulence factor of fungal and bacterial pathogens and as a signaling compound in disease resistance (van Loon et al., 2006a). However, the role of this hormone in the postharvest disease resistance of fruit is still unknown. In both ripe and unripe tomato fruit, *B. cinerea* and *C. gloeosporioides* infections induce the ethylene biosynthesis pathway; transcription factors like non-ripening (NOR), ripening-inhibitor (RIN) and

never-ripe (NR); and the ethylene-regulated defense genes (Alkan et al., 2015; Blanco-Ulate et al., 2013). In citrus fruit, ethylene production has been found to increase with *P. digitatum* infection, and this hormone is synthesized by both the fruit and the pathogen (Achilea et al., 1985a; Achilea et al., 1985b). Moreover, the fact that many genes induced in citrus fruit upon *P. digitatum* attack are also up-regulated by ethylene highlights the role this hormone plays in the defense response of citrus to this pathogen (González-Candelas et al., 2010a).

During recent years, a growing number of studies have sought to identify cultivars with a high level of resistance to pathogens. This increase can be attributed to the fact that more and more breeding programs are seeking out new tools for the early selection of seedlings with enhanced resistance to pathogens. Several studies have provided data on genetically determined levels of resistance to *B. cinerea* in strawberry (Bestfleisch et al., 2015) and to *P. expansum* in in apple cultivars from Norway and Sweden (Ahmadi-Afzadi et al., 2015; Tahir et al., 2015); Mexico (Sandoval et al., 2014); and Kazakhstan (Jurick et al., 2011). Overall, all these studies should be used in breeding programs for selecting apple cultivars with a high level of resistance to fungal pathogens. In peach breeding programs, Pacheco et al. (2014) and Martínez-García et al. (2013) have identified QTLs in a few genomic regions underlying brown rot response traits and providing a starting point for the development of marker-assisted selection for increased resistance to this pathogen. Preliminary results from an apple breeding program have already made it possible to identify QTLs for blue mold resistance in *Malus sieversii* (Norelli et al., 2014).

6. **Concluding remarks and future perspectives**

Losses due to the postharvest decay of fruits still represent a major concern both from an economic and food safety point of view. Despite the great effort that has gone into

developing new and safer alternatives to chemical fungicides, these compounds continue to represent the main tool for controlling the major postharvest pathogens. Most of the alternative treatments developed so far have some limitations that impede their use as stand-alone treatments. Although the possibility of combining different treatments within an integrated pest management strategy seems to be the predominant trend, it is clear that we need further developments in this field. Moreover, these developments will probably arise from deeper knowledge of the fruit-pathogen-environment interactions at the physiological, biochemical and molecular level.

In this review we have focused on the topics currently being investigated in both major postharvest pathogens and main fruit crops. Among the major postharvest phytopathogenic fungi, *B. cinerea* is the best characterized from a molecular point of view, a fact that reflects the importance of this fungus as a general plant pathogen. In recent years, however, extensive research has also been conducted on other important postharvest pathogens, such as *P. expansum*, *P. digitatum*, *C. gloeosporioides* and *A. alternata*. Interestingly, most of these fungi are necrotrophs that kill the cell to obtain nutrients. For a long time, these fungi were thought to rely on CWDEs and other extracellular hydrolases, such as proteases, as the major virulence factors. However, the characterization of gene knockout mutants indicates that in most instances all these enzymes are just necessary for full virulence, but they are not essential for pathogenesis. A similar situation is being uncovered with pathogen enzymes involved in ROS production/detoxification. These results are probably not so surprising, however, because many of these enzymes belong to families with redundant enzymes, and the loss of one member of the family can be compensated for by another enzyme that carries out a similar biological function. Although the biological functions of the majority of the virulence

genes identified and characterized by molecular biology techniques are still not clear, there is increasing optimism supported by recent experimental evidence (An et al., 2016; Noda et al., 2010; Siegmund et al., 2015; Zhang et al., 2014b).

One of the research topics that is probably gaining the most attention in many pathogenic fungi, and which we think is likely to gain traction in the near future in postharvest pathogens, is the study of effectors: pathogen molecules that modulate host defense responses favoring the development of the pathogen. Although most effectors characterized so far are small cysteine rich secreted proteins, there are new players entering the scene. Small RNAs are emerging as very important regulators of gene expression, and not only in the organism in which they are synthesized, since they can be translocated to the host cells where they manipulate defense signaling pathways favoring pathogen infection. The study of small RNAs will probably open new perspectives in the study of fruit-pathogen interactions.

In order to be able to infect, pathogens have to adapt to the environmental conditions found within the host. As we have seen, these factors, such as ambient pH, light or the presence of exogenously added substances, also play a fundamental role in the outcome of fruit-pathogen interactions. The manipulation of environmental conditions thus constitutes another tool that can be exploited for the control of postharvest diseases. Furthermore, other environmental factors deserve further research, like the manipulation of nutrient availability or increasing the concentration of compounds that either promote fruit resistance or that are toxic to the pathogen.

Some of these environmental factors are more related to the fruit than to the pathogen. In this context, we are just starting to answer some old questions, like why ripe and unripe fruits show differential susceptibility to pathogens. The studies described in this review

are providing new perspectives that warrant further research to elucidate, for example, the role of lipids in fruit defense or the interplay among different defense signaling pathways. These mechanisms are additional to other fruit defense mechanisms that have received more attention in the fruit-pathogen interaction, like the role of ROS, PR proteins, lignification or phenylpropanoids metabolism. Besides inducing resistance, there are clearly two major strategies for increasing the resistance of fruits to pathogens. One involves the genetic manipulation of the fruit crop following similar strategies already used to increase resistance in whole plants. The other involves breeding programs that aim to introgress resistance QTLs from wild varieties into commercial germplasms. Such programs are already underway in important crops like apple, peach and strawberry. Hopefully these long-term initiatives will provide new varieties with improved resistance against major postharvest pathogens.

Finally, we want to emphasize that the major challenge that we will probably face in the coming years in the molecular characterization of fruit-pathogen interactions is the full development of the new "omics" techniques to help us unravel both pathogenicity/virulence factors in the pathogen and resistance/susceptibility mechanisms in the fruit. The implementation of these technologies will offer an unprecedented level of knowledge on fruit-pathogen interactions that can establish new bases for the development of control strategies, which is our ultimate goal as postharvest pathologists.

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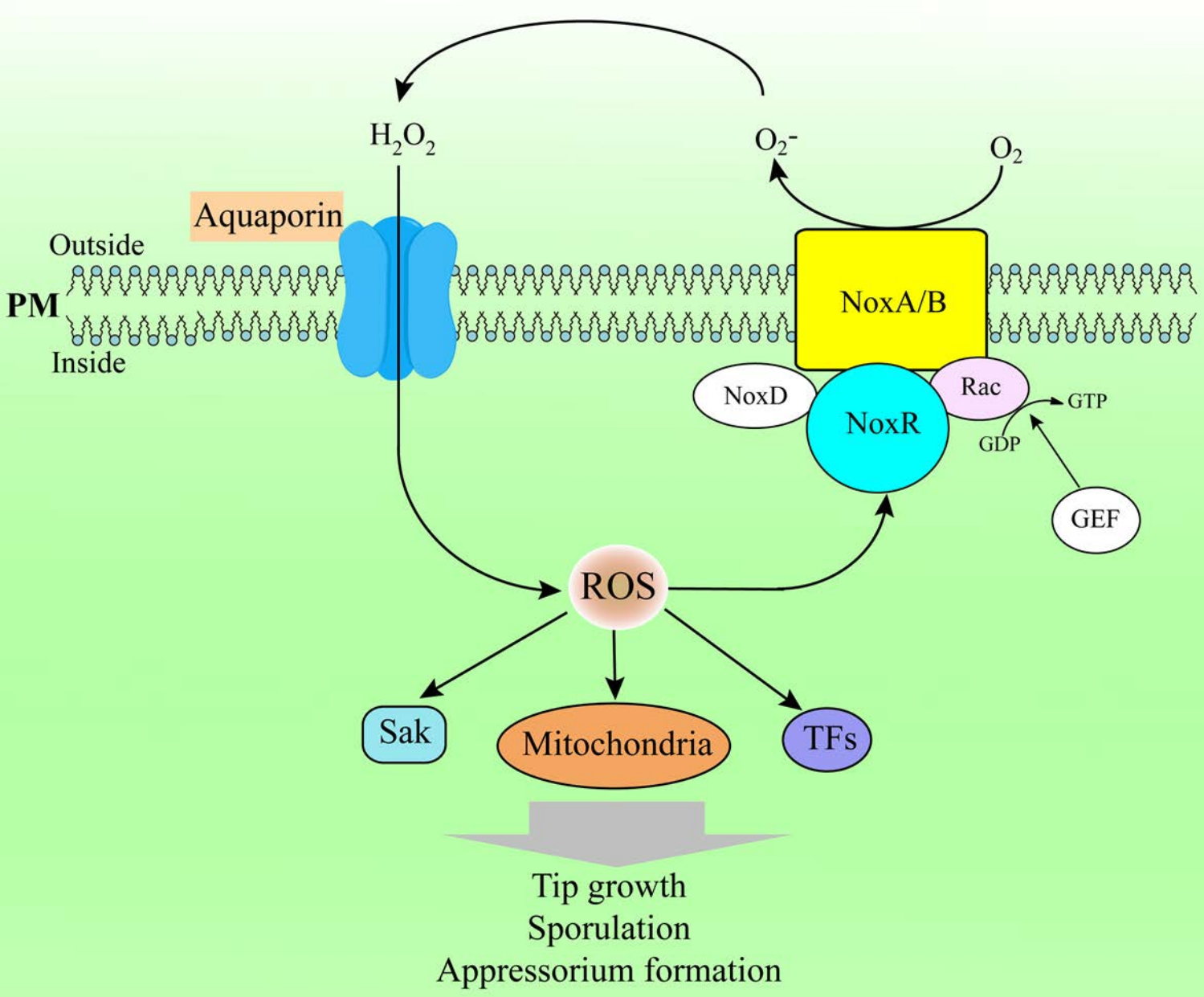
Figure legends

Figure1. Model for ROS in the regulation of differentiation and virulence in fungi. The NADPH oxidase (Nox) complexes, which are composed of several subunits (NoxA/B, NoxR, NoxD, Rac), are the most important enzymatic ROS generating system. The

Nox complexes use NADPH as an electron donor and transport electrons through the plasma membrane (PM) to reduce oxygen to superoxide. The superoxide outside of cells can be converted into H_2O_2 and transported into cells by certain channels, such as aquaporins. Mitochondrion is one of the action targets of intracellular ROS. Moreover, ROS, as signaling molecules, can regulate the function of Nox complexes, phosphorylation of the MAP kinase Sak, and the expression of oxidative stress response genes through transcription factors (TFs). Finally, the mycelial growth, sporulation and appressorium formation were regulated by the complicated ROS regulatory network.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