



# Reactive oxygen species: A generalist in regulating development and pathogenicity of phytopathogenic fungi



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## ABSTRACT

Reactive oxygen species (ROS) are small molecules with high oxidative activity, and are usually produced as byproducts of metabolic processes in organisms. ROS play an important role during the interaction between plant hosts and pathogenic fungi. Phytopathogenic fungi have evolved sophisticated ROS producing and scavenging systems to achieve redox homeostasis. Emerging evidences suggest that ROS derived from fungi are involved in various important aspects of the development and pathogenesis, including formation of conidia, sclerotia, conidial anastomosis tubes (CATs) and infectious structures. In this mini-review, we summarize the research progress on the redox homeostasis systems, the versatile functions of ROS in the development and pathogenesis of phytopathogenic fungi, and the regulation effects of exogenous factors on intercellular ROS and virulence of the fungal pathogens.

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## 1. Introduction

It is estimated that global agricultural production need to increase by 60–110% by 2050 to meet the increasing demands

caused by population growth and the increased consumption of meat and dairy [1,2]. More seriously, phytopathogenic fungi cause significant losses in global crop production every year, which poses a major threat to the world's food security [3,4]. Therefore, it is very important to control crop diseases to reduce yield loss. The in-depth understanding of the pathogenesis mechanism of pathogenic fungi will help to formulate more effective control strategies for plant disease.

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During the past decades, great efforts have been made to unravel the pathogenesis of phytopathogenic fungi. Previous studies have shown that the pathogenicity of phytopathogenic fungi can be regulated at multiple levels, such as environmental conditions [5,6], protein secretion [7], signal transduction [8], gene transcription [9,10], etc. It is well known that, at the initial stage of pathogen infection, plant cell can rapidly accumulate abundant of reactive oxygen species (ROS) around the infection site, called as “oxidative burst”, which is considered as the primary disease resistance response [11,12]. In addition, ROS, together with  $Ca^{2+}$  and electrical signals, can also be used as secondary messengers to regulate systematic acquired resistance (SAR) of plant [13,14]. The hypersensitive response of host can limit the spread of biotrophic pathogens. By construct, the ROS-induced cell death is beneficial to necrotrophic pathogens, which feed on the degradation products of plant cells [15]. ROS produced by fungal pathogens includes singlet oxygen ( $^1O_2$ ) superoxide anion ( $\bullet O_2^-$ ), hydroxyl radical ( $\bullet OH$ ) and hydrogen peroxide ( $H_2O_2$ ), and accumulate at the hyphal tips during the infectious process [15]. Moreover, ROS are usually produced as byproducts of some metabolic processes in organisms and are converted from oxygen molecules through electron transfer reactions, which usually take place in mitochondria, peroxisomes and NADPH oxidase complex [16], and can react with many macromolecules, such as protein, DNA and lipid, leading to cell apoptosis or death [17,18]. As in animals and plants, fungi have also evolved a set of complicated producing and scavenging systems of ROS to balance the cellular redox state. A growing body of evidence suggests that ROS derived from pathogenic fungi play critical roles in the regulation of development and pathogenicity. In particular, recent studies demonstrate that many components of ROS producing systems (such as NADPH oxidase complex) have sophisticated regulatory mechanisms in various aspects of the biological processes in many phytopathogenic fungi, including some important fungal pathogens of crops, *Magnaporthe oryzae* and *Botrytis cinerea* [19–22].

Here, we will briefly summarize the research progress of ROS in phytopathogenic fungi in the following aspect: 1) the cellular

redox homeostasis systems; 2) the transport and distribution of ROS; 3) the functions of ROS in development and pathogenicity; 4) regulation effects of exogenous factors on intercellular ROS.

## 2. The producing and scavenging mechanisms of ROS in phytopathogenic fungi

In order to maintain the balance of redox state, phytopathogenic fungi have evolved a set of ROS producing and scavenging mechanisms (Fig. 1). Among the fungal ROS producing systems, NADPH oxidase (Nox) is most widely characterized. Nox complex is composed of several subunits and usually localize at plasma membrane or endoplasmic reticulum membrane, and transport electrons through membranes reducing oxygen molecule to superoxide anion ( $\bullet O_2^-$ ) using NADPH as electron donor [23]. Then, superoxide dismutase (SOD) catalyze  $\bullet O_2^-$  into  $H_2O_2$ . In filamentous fungi, there are two distinct subfamilies of Nox, NoxA and NoxB, which are the homologs of gp91<sup>phox</sup> in mammals [24]. In some fungi, such as *M. oryzae*, *Podospora anserina* and *Aspergillus terreus*, a third Nox (NoxC) was characterized, which contains putative calcium binding EF-hand motifs ( $Ca^{2+}$  binding) at the N-terminus, a feather of human Nox5 or plant RBOHs [24]. In addition to the catalytic subunits, Nox complex also contains intracellular regulatory subunits and the subunits for stabilizing structure. Adapter protein is required for the function of Nox complex, and NoxD, which is the homolog of the adaptor protein p22<sup>phox</sup> in mammals, has been identified in some plant pathogenic fungi, such as *Botrytis cinerea*, *Podospora anserina* and *M. oryzae* [22,25–27].

In *B. cinerea*, BcNoxD can directly interact with catalytic subunit NoxA, and another transmembrane protein BcPls1 performs a similar function to BcNoxD in the NoxB complex [21]. Apart from the membrane-standing subunits, other components are also necessary to maintain the activity of Nox complex, including the regulatory subunit NoxR, small GTPase Rac and Rho [20,21,28,29]. The interaction between regulatory subunit and catalytic subunit take

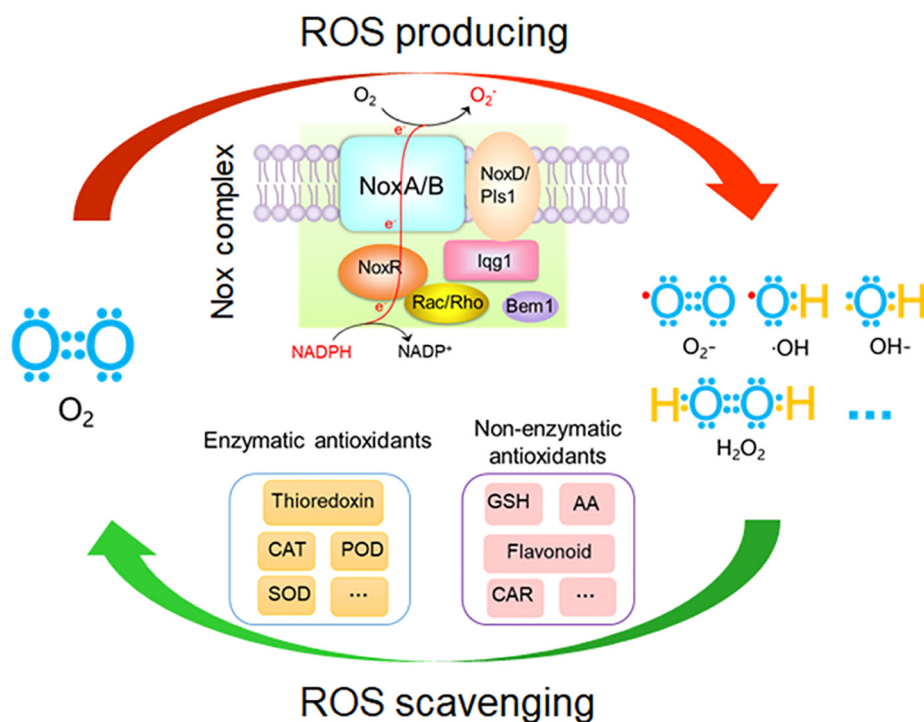


Fig. 1. The producing and scavenging systems of ROS in pathogenic fungi.

place at the cytoplasmic side of membrane [30,31]. In most cases, both NoxA and NoxB can be regulated by NoxR and Rac. The mutant of NoxR mutant always shows an additive phenotype of the mutant of NoxA and NoxB, suggesting that NoxR is the regulatory subunit of both NoxA and NoxB [20,32]. In some fungi, the small GTPase cdc42 and scaffold protein Bem1 were also identified as the subunits of the Nox complex [33,34]. The deletion of Nox genes in some fungi does not reduce the ROS level, and even increases the accumulation of intercellular ROS [19,35,36]. This suggests that there are other alternative ROS producing systems in phytopathogenic fungi besides Nox complex. The mitochondria also produce continuous levels of  $\bullet\text{O}_2^-$  at complex I and III of electron transport chain, and have been considered to produce more ROS in quantitative terms than Nox in most cell types [37,38]. In addition, other enzymatic systems of phytopathogenic fungi are contributed to the ROS production in form of the byproduct of redox reaction, including laccases, galactose oxidases, quinone reductases and glucose oxidase [36,39,40,41].

Fungi possess several ROS scavenging systems to neutralize excessive ROS originating from normal physiological processes or environmental stresses. The antioxidant systems are mainly divided into non-enzymatic type and enzymatic type [15]. The major non-enzymatic antioxidant is glutathione, a tripeptide  $\gamma$ -L-glutamyl-L-cysteinyl-glycine. The free thiol group of this small soluble tripeptide can transfer the electrons of oxygen radicals, and then two reduced glutathione molecules (GSH) form an oxidized molecule (GSSH) through a disulfide bond [42]. The addition of exogenous GSH can significantly enhance oxidative tolerance and biocontrol efficiency of antagonistic yeast against *Penicillium expansum* [43]. In addition to GSH, some other organic compounds in fungi also have antioxidant properties, such as ascorbic acid, carotenoids, flavonoids and alkaloids [44]. Thioredoxin proteins and their respective reductases are the most common enzymatic ROS scavenging system. The thioredoxins served as the reducing agents of ROS by forming disulfide bonds between two inherent cysteines. In *B. cinerea*, there are two thioredoxins (BcTrx1 and BcTrx2) and one thioredoxin reductase (BcTrr1), and the deletion of *BcTrx1* and *BcTrr1* led to highly sensitive to oxidative stress [45]. Besides the thioredoxin system, catalases, superoxide dismutases and peroxidases are also involved in the scavenging of ROS in pathogenic fungi [8,30]. SOD can convert  $\bullet\text{O}_2^-$  to  $\text{H}_2\text{O}_2$ , and CAT and POD sequentially degrade  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  and  $\text{O}_2$ .

### 3. The intracellular distribution and transport of ROS in phytopathogenic fungi

The levels and distribution of ROS in cells is a dynamic process depending on different developmental stages. ROS are usually generated at different sites of cell, such as cytosol, vacuoles, peroxisomes and mitochondria. In the growing hyphae, mitochondria are the main source of intercellular ROS, and the distribution of ROS shows obviously polarity at the apical tip of hyphae [46]. Similarly, in the appressorium formation stage of infectious process,  $\bullet\text{O}_2^-$  also accumulates inside the hyphal tip [47].

The small GTPase Rho3 is closely related to the polar distribution of intercellular ROS in *B. cinerea*. The deletion of *rho3* can impair the polarity of ROS distribution at the hyphal tip and decrease the virulence of *B. cinerea* to apple and tomato fruits [28]. BcnoxR, as the regulatory subunit of Nox complex, significantly affects ROS distribution in cell and virulence of fungal pathogens. The  $\Delta$ bcnor mutant shows reduced grow and sporulation, and impaired virulence of *B. cinerea* to apple, strawberry and tomato fruits [32]. It means that the distribution of ROS at the hyphal tip is important for the polar growth of hyphae and virulence of pathogenic fungi.

Aquaporins (AQPs) are integral membrane proteins and play important role in regulating water or glycerol homeostasis in unicellular and multicellular organisms by mediating rapid water transport across biological membranes. The aquaporins of fungi can be subdivided to five classes, including two groups of classical aquaporins and three groups of aquaglyceroporins [48]. There are eight AQPs in *B. cinerea*, among which only AQP8 has been proved to be involved in ROS distribution and transmembrane transport [49]. Heterologous expression of AQP8 in yeast increased the uptake of exogenous  $\text{H}_2\text{O}_2$ , indicating that AQP8 mediates transmembrane transport of ROS in cell (Fig. 2). Moreover, the expression of *BcnoxR* was significantly affected by AQP8, suggesting that the transmembrane transport of ROS mediated by AQP8 is closely related to Nox in regulating the cellular redox homeostasis [49].

### 4. ROS regulating development and virulence of phytopathogenic fungi

Function analysis have unraveled that the components of ROS producing and scavenging systems are involved in the developmental processes and virulence of fungal pathogens (Fig. 2). In phytopathogenic fungi, conidia are the main source of transmission and infection, and sclerotia are important stress-resistant tissue. Both of them are regulated by intercellular ROS. Oxidative stress can induce the formation of sclerotia of *Sclerotium rolfsii* [50]. In *B. cinerea*, the subunits of Nox complex, including NoxA, NoxB, NoxR, NoxD and Pls1, were all responsible for sclerotia formation; meanwhile, NoxD, Rac, Rho3 and Bem1 are related to the conidial production [8,46]. In *Alternaria alternata*, the inactivation of *NoxA*, *NoxB* or *NoxR* genes will reduce conidiation by 95% [51]. Furthermore, some knockout mutants of Nox subunits in plant pathogenic fungi showed slower growth rate [28,52], implying that ROS is a critical regulator of vegetative and reproductive developmental processes of the fungi. Recent studies in some model fungi have indicated that ROS are actively involved in the formation of conidial anastomosis tubes (CATs). CATs are short germ tube that can fuse with each other, which is a self-fusion mechanism in fungi. ROS produced by Nox complex are considered to be involved in this cell fusion process. In *Neurospora*, ROS might directly regulate the cell–cell recognition at the hyphal tip [53]. The catalytic subunits NoxA and regulatory subunit NoxR of *B. cinerea* have proved to be responsible for the fusion process of CATs [20,54]. Subsequently, Siegmund et al., (2015) found that NoxA must be exported from the endoplasmic reticulum for CAT formation, and NoxD can directly interact with NoxA to affect the CAT formation in *B. cinerea* [26]. In addition, ROS can modulate the circadian rhythm by influencing the redox homeostasis of pathogenic fungi [55].

ROS play a critical role during the interaction between fungal pathogens and plant hosts [16]. Once the plant tissue is attacked by pathogenic fungi, it will quickly accumulate a large amount of ROS around the infection site as the initial resistant response [31]. The oxidative burst can effectively inhibit the colonization of biotrophic pathogens; while the necrotrophic pathogens, such as *B. cinerea* and *Leptosphaeria maculans*, can benefit from the host cell death caused by ROS accumulation to facilitate its infection, because they can only survive in dead cells [15]. Apart from the ROS produced by plant cell, ROS produced within fungal cells are also rapidly accumulated in the hyphal tips or around the penetrated cell wall during the infectious process [15]. This suggests that the ROS derived from phytopathogenic fungi are actively involved in the infection process. In *B. cinerea*, both catalytic subunits NoxA and NoxB have great impact on pathogenicity, but there is a distinct difference between their function. NoxB as well

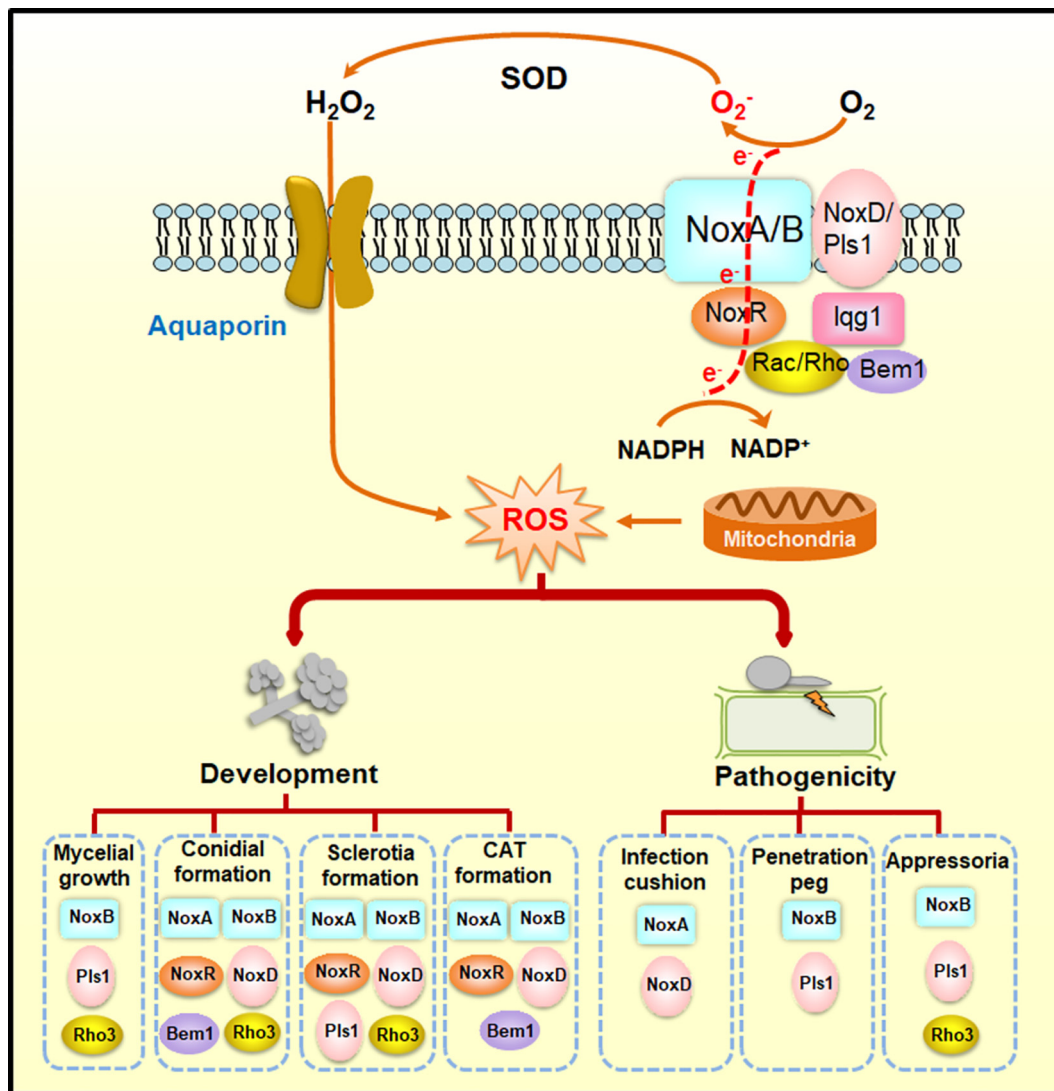


Fig. 2. The functions of ROS in regulating the development and pathogenicity of pathogenic fungi.

as its adaptor protein Pls1 are necessary for the development of appressoria and involved in the formation of primary lesions. Instead, NoxA and its adaptor protein NoxD are responsible for the formation of infection cushion and regulate the colonization process following primary infection [15]. The regulatory subunit NoxR showed an additive effect of NoxA and NoxB in regulating the pathogenicity of *B. cinerea* [20]. In *M. oryzae*, the rapid accumulation of ROS during infection is closely related to the differentiation of appressorium and penetration peg, thereby influencing the virulence [19]. Nox2 was involved in the septin-mediated repolarization of appressorium, but Nox1 was required for the maintenance of the polarized growth. Double deletion of Nox1 and Nox2 in *M. oryzae* led to non-pathogenicity [19]. Interestingly, both in *B. cinerea* and *M. oryzae*, deletion of Nox did not affect the ROS level [19,20], implying that alternative ROS producing system may be contributed to the infectious process of phytopathogenic fungi. Giesbert et al., (2008) demonstrated that Nox could not affect the penetration, but Cpnox1 was required for the following colonization process in the biotrophic pathogen *Claviceps purpurea*, which do not form specialized infection structures [56]. Similarly, NoxA, NoxB and NoxR show important effect on the regulation of virulence of *A. alternata* in citrus [51]. However, in *A. alternata* Japanese pear pathotype, only NoxB was essential for the pathogenicity,

while the deletion of NoxA had no obvious effect on pathogenicity [57]. In recent, Zhao et al., (2016) reported that the VdNoxB and VdPls1 in *Verticillium dahliae*, a pathogenic fungus causes wild disease in many crops, could regulate penetration peg formation during the initial colonization of cotton roots [58]. These results indicate that the subunits of Nox complex affect virulence of phytopathogenic fungi via mediating the differentiation and development of specialized infection structures of the fungi. Meanwhile, the contributions of Nox genes to pathogenicity differ in different fungal species, even in different pathotypes.

### 5. Exogenous factors affecting ROS production of fungi

In view of the critical roles of ROS in regulating the development and virulence of phytopathogenic fungi, exogenous factors are widely used to control the plant diseases, because they have a target to ROS in the fungal cells. Borates are essential micronutrients of plant, and have been proved to be effective in controlling diseases caused by *Penicillium expansum* in apple [59] and by *Colletotrichum gloeosporioides* in mango fruit [60]. *P. expansum* treated with 1% borate showed a rapid accumulation of intercellular ROS and protein carbonylation, leading to the decrease of virulence of the pathogen [59]. Up on the exposure to borate, abundance of

two critical antioxidant enzymes, catalase and glutathione S-transferase, were significantly decreased in *P. expansum*, which led to the impaired ROS scavenging capacity [59]. Similarly, borate treatment reduces the anthracnose in mango fruit by inducing the ROS generation and mitochondrial degradation in the spores of *C. gloeosporioides* [60]. Recent research has shown that some bio-source antimicrobial agents (such as cinnamic acid, methyl thujate, epsilon-polylysine, honokiol etc.) can inhibit the virulence of phytopathogenic fungi by inducing the excessive accumulation of intercellular ROS and disturb the redox homeostasis [61–64]. Methyl thujate treatment could significantly induce the expression of the Nox genes in *B. cinerea*, including *BcNoxB*, *BcNoxD*, *BcNoxR*, *BcCdc24*, *BcPls1*, *BcRac1* and *BcBem1*, which accelerated the production of ROS [62]. Apart from the chemical substances, heat treatment (40 °C for 5 min) has been reported to have inhibiting effect on spore germination and germ tube elongation of *Monilinia fructicola*, because of induction of ROS in cells of the fungal pathogen [65]. Honokiol and heat treatment will led to significant decline of mitochondrial membrane potential in pathogenic fungi, suggesting mitochondrial dysfunction and probable release of cytochrome C from mitochondria [64,65]. Biological control of plant diseases by antagonistic yeasts is a promising alternative to chemical fungicides. However, biocontrol efficacy of the yeasts is closely related to their antioxidant ability. Some exogenous antioxidants or antioxidant inducers (such as glutathione and glycine betaine) have been found to significantly improve the biocontrol efficacy of antagonistic yeasts against fungal pathogens, because these substances can enhance the tolerance of the yeasts to oxidative stress [43,66].

## 6. Conclusions and future prospects

ROS are ubiquitous in living cells and play an important role in all fungus-plant interactions, mostly as signaling components. Phytopathogenic fungi have developed efficient ROS scavenging systems that are under complex regulatory control, but less known so far about ROS sensing systems. In phytopathogenic fungi, the ROS producing systems mainly include NADPH oxidase (Nox) complexes and mitochondria; while the ROS scavenging systems have the non-enzymatic GSH-GSSH and enzymatic thioredoxin. Nox complexes are the most common enzymatic producer of ROS, and their subunits are involved in various aspects of development and virulence in phytopathogenic fungi. ROS regulate the virulence of phytopathogenic fungi via affecting the development of conidia, sclerotia, CATs and infectious structures of the fungi. Considering the important of ROS involved in the development and virulence of phytopathogenic fungi, the redox homeostasis system can be used as an important target of many exogenous factors to control the virulence of the fungi. Some exogenous substances have been proved to inhibit virulence of phytopathogenic fungi by mediating polar distribution of ROS at the hyphal tip. The integral membrane protein aquaporin (AQP8) may mediate the transmembrane transport of ROS in *B. cinerea*.

In a word, ROS have aroused extensive research interest in phytopathogenic fungi, and the great progress in understanding the intracellular production of ROS, and the function in the regulation of development and virulence of the fungal pathogens has been made in recent years. However, there are still many unknown functions and mechanisms of ROS in phytopathogenic fungi to deeply study, including 1) what are the specific mechanisms of action of ROS in different phytopathogenic fungi? since there is difference in the action mechanisms of ROS in necrotroph and biotroph; 2) what are their targets of ROS in the regulation of developmental processes and virulence of phytopathogenic fungi? 3) why are ROS scavenging systems not related to virulence of phy-

topathogenic fungi in most cases? 4) what are Intracellular transport pathways and special transporters of ROS? Since transport mechanism of ROS in phytopathogenic fungi is very limited. With the development of research techniques and methods, these issues will be revealed in the future.

## Author contributions

S-P. T conceived the review article and revised the manuscript. Z-Q. Z wrote the manuscript. Y. C, B-Q. L and T. C provided data and conceived the figures. All authors have approved the final article.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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