Published in "Phytochemistry 112: 54–62, 2015"
which should be cited to refer to this work.

Reactive oxygen species and plant resistance to fungal pathogens

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Reactive oxygen species (ROS) have been studied for their role in plant development as well as in plant immunity. ROS were consistently observed to accumulate in the plant after the perception of pathogens and microbes and over the years, ROS were postulated to be an integral part of the defence response of the plant. In this article we will focus on recent findings about ROS involved in the interaction of plants with pathogenic fungi. We will describe the ways to detect ROS, their modes of action and their importance in relation to resistance to fungal pathogens. In addition we include some results from works focussing on the fungal interactor and from studies investigating roots during pathogen attack.

1. Introduction

So-called reactive oxygen species (ROS) include various forms of reduced and chemically reactive molecules such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) or hydroperoxyl radical ($HO_2^{\cdot}$). Up to the 1980s the synthesis as well as the detoxification of ROS had already attracted many researchers and ROS were much studied for their role in plant development (Elstner, 1982; Swanson and Gilroy, 2010; Tian et al., 2013).

In 1983, Doke reported a production of $O_2^{\cdot-}$ during an incompatible interaction of potato with the oomycete *Phytophthora infestans* (Doke, 1983). This observation set forth a considerable wave of studies on the production of ROS in whole plants or in suspension cells confronted with live pathogens or various elicitors. It has become apparent that ROS are an integral response to both biotic and abiotic stress. A large number of reviews have been dedicated to this topic (Apel and Hirt, 2004; Baker and Orlandi, 1995; Barna et al., 2012; Baxter et al., 2013; Foyer and Noctor, 2013; Laloi et al., 2004; Mehdy, 1994; Miller et al., 2008; Mittler, 2002; Mittler et al., 2011; O'Brien et al., 2012a; Sutherland, 1991). In this article, we

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will briefly review the available methods to detect the formation of ROS in plant tissue. We will then discuss the different possible modes of action of ROS, as their deployment is among the early reactions after the perception of pathogen-, microbe- or damage-associated molecular patterns (PAMPs, MAMPs or DAMPs) by pattern recognition receptors (Boller and Felix, 2009; Torres, 2010; Torres et al., 2006). Finally, we will review the more recent evidence establishing the link between ROS production and resistance to fungal pathogens both in leaves and the roots.

2. Detection of ROS

Many methods are used to detect the accumulation of ROS. They are based on histochemical staining, fluorescence, luminescence, electron paramagnetic resonance (EPR) spectroscopy or ROS sensors (Table 1). Fluorescent probes, $CeCl_3$ and ROS sensors are also used for a subcellular localisation of ROS. A difficulty with the detection of ROS lies in their relative short life-times combined with the ability of living cells to scavenge ROS. Furthermore, tissue damage or disruption during tissue handling might generate ROS artefacts. The lack of probes with a high selectivity is another hurdle. In other words, no probe is guaranteed to work for a given tissue under given conditions. Given these difficulties, researchers engaging in the detection and localisation of ROS in plant tissue are therefore advised to make the necessary controls and preliminary tests to determine the validity of the probes they are using. This includes using more than one method to support their conclusions. In the next section, we have briefly summarised the most common approaches used and indicated some recent publications where they have been applied. The reader is referred to several useful reviews on methodological aspects and associated difficulties and limitations concerning ROS detection (Freinbichler et al., 2011; Nauseef, 2014; Winterbourn, 2014; Zulfugarov et al., 2011).

H_2O_2 can be detected with the histochemical stain 3-3′ diaminobenzidine (DAB) that forms instantly a brownish polymer in presence of H_2O_2 and peroxidase (Thordal-Christensen et al., 1997). DAB staining has been often used to visualise the generation of H_2O_2 *in planta* (Asai et al., 2010; Dubreuil-Maurizi et al., 2010; Kobayashi et al., 2012; L'Haridon et al., 2011; Liao et al., 2012; Rojas et al., 2012; Simon et al., 2013; Torres et al., 2005; Yokawa et al., 2011; Zhang et al., 2012). The Amplex Red hydrogen peroxide/peroxidase activity assay also allows quantifying H_2O_2 concentrations and consists in a non-fluorescent molecule that is oxidised by H_2O_2 and becomes fluorescent in presence of peroxidase. Recent examples of this method applied to plants are cited hereafter (Shin and Schachtman, 2004; Zhang et al., 2012). An analogous method to quantify H_2O_2 uses 3-methylbenzothiazoline hydrazine that reacts in presence of peroxidases (Malolepsza and Rozalska, 2005). Assays with ferrous ion oxidation (FOX) are based on the spectrophotometrical detection of peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} ions that forms a complex with xylenol orange. The FOX method was used on cultured suspension cells (Boubakri et al., 2013; O'Brien et al., 2012b) or on incubation medium of leaf explants (Bellincampi et al., 2000). Other methods to determine H_2O_2 spectrophotometrically in plant samples include the use of resorcinol/titanium oxalate (Becana et al., 1986) or ABTS (2,2′-Azino-bis-(3-ethylbenthiazoline-6-sulfonate)) (formation of blue colour) (Messner and Boll, 1994). The cytochemical staining using cerium (III) chloride ($CeCl_3$) is used for a subcellular localisation of H_2O_2. The reaction between $CeCl_3$ and an excess of H_2O_2 generates electron-dense deposits of cerium perhydroxides that can be observed using transmission electron microscopy (Bestwick et al., 1998; Fester and Hause, 2005; Lherminier et al., 2009; Simon et al., 2013; Xia et al., 2009).

To visualise superoxide oxygen anions in the plant tissue, the histochemical stain nitroblue tetrazolium (NBT) is frequently used.

Table 1
Summary of different techniques that are used to detect ROS in plants.

Product	Reactive oxygen species	Detection
3–3′ diaminobenzidine (DAB)	H_2O_2	Histochemical
Amplex red	H_2O_2	Spectrophotometrical
3-Methylbenzothiazoline hydrazine	H_2O_2	Spectrophotometrical
Ferrous ion oxidation (FOX)	H_2O_2	Spectrophotometrical
Ti^{4+} method	H_2O_2	Spectrophotometrical
ABTS	H_2O_2	Spectrophotometrical
Cerium (III) chloride ($CeCl_3$)	H_2O_2	Cytochemical
Nitroblue tetrazolium (NBT)	$O_2^{\cdot-}$	Histochemical
Dihydroethidium (DHE)	$O_2^{\cdot-}$	Fluorescence
2-Deoxyribose (DOR)	$OH^{\cdot}$	Spectrophotometrical
Spin trapping electron paramagnetic resonance spectroscopy (EPR)	Oxygen free radicals	Spectroscopy
Dansyl-based fluorescence sensors	$O_2^{\cdot-}$; $^1O_2$	Fluorescence
Singlet Oxygen Sensors Green (SOGS)	$^1O_2$	Fluorescence
Fluorescein diacetate	ROS	Fluorescence
Dihydrorhodamine123	ROS	Fluorescence
Luminol	ROS	Chemiluminescence

Yellow, water-soluble NBT is reduced by superoxide radicals to blue, water-insoluble formazan (Grosskinsky et al., 2012; Jabs et al., 1996; Kawarazaki et al., 2013; L'Haridon et al., 2011; Liao et al., 2012; Wang and Higgins, 2006; Xia et al., 2009). Superoxide anion radicals can also be detected using dihydroethidium (DHE), a cell-permeable blue fluorescent stain that forms red fluorescent oxyethidium upon oxidation and intercalates with nucleic acids (see recent applications in Lehotai et al., 2011; Mai et al., 2013; Pető et al., 2013). The hydroxyl radicals can be quantified using 2-deoxyribose (DOR), a scavenger and a probe as exemplified in the study on *Botrytis cinerea*-infected tomato leaves by Malolepsza and Rozalska, 2005. DOR is sensitive to hydroxyl radicals and thiobarbituric acid-reactive degradation products are formed that can be determined spectrophotometrically (von Tiedemann, 1997). Electron paramagnetic resonance spin trapping spectroscopy (EPR) allows detection of oxygen free radicals or other species with unpaired electrons (reviewed by Bacic and Mojovic, 2005). Diamagnetic spin traps are used that react with free radicals and form an adduct that can be detected using EPR spectroscopy. For example, this method was used to detect the singlet oxygen in thylakoid membranes under photoinhibitory conditions or UV stress (Hideg et al., 1994, 1995). The singlet oxygen and superoxide anion radicals can be also detected and localised using dansyl-based fluorescence sensors such as DanePy or HO-1889-NH (Hideg et al., 2002) as well as a Singlet Oxygen Sensors Green (SOGS) (Flors et al., 2006; Plancot et al., 2013).

Fluorescein diacetate (H_2DCF-DA, CM-H_2DCF-DA) and dihydrorhodamine 123 are among the commonly used fluorescent probes to detect a broad spectrum of ROS. They consist in non-fluorescent molecules that become fluorescent when oxidised by ROS, and the emitted fluorescence can be observed by fluorimetry and/or by fluorescent microscopy, an advantage of such probes (Benikhlef et al., 2013; Bulgakov et al., 2012; Fester and Hause, 2005; Guo et al., 2010; Kolla et al., 2007; L'Haridon et al., 2011; Li et al., 2007; Liu et al., 2010; Ma et al., 2013; Peleg-Grossman et al., 2012; Plancot et al., 2013; Tada et al., 2004; Wen et al., 2008; Ye et al., 2013). Luminol or luminol analogues are sensitive chemiluminescent probes used to quantify a relative intensity of ROS by counting the emitted light with a luminometer, CDD camera or a scintillation counter (Dubreuil-Maurizi et al., 2010; Flury et al., 2013; Kunz et al., 2006; L'Haridon et al., 2011; Mersmann et al., 2010). Finally, it is possible to determine the redox potential of the glutathione pool in a high spatial and temporal resolution using various redox-sensitive green fluorescent proteins (for example roGFP) encoded in the test plant. The method requires transient

or permanent plant transformation and has already been used in many cases (Beneloujaephajri et al., 2013; Heller et al., 2012; Jubany-Mari et al., 2010; Lehmann et al., 2009; Rosenwasser et al., 2011).

3. The multiple functions of ROS

ROS released in plants during an interaction with microbes can affect both partners. Fungal pathogens as well as other invading microorganisms are exposed to the oxidative stress generated by ROS and they have evolved multiple ways to scavenge ROS using for example small molecules (glutathione, ascorbic acid, flavonoids, alkaloids and carotenoids) that will be oxidised by ROS, as well as detoxifying enzymes (superoxide dismutase, peroxidase, catalase, peroxiredoxins).

One approach to evaluate the importance of ROS for plant defences is to interfere with the mechanisms deployed by pathogens that protect them against ROS. Recently, the transcription factor Moatf1 homologous to the yeast ATF/CREB that regulates the oxidative stress response was identified in *Magnaporthe grisea*. Targeted gene deletion of *Moatf* results in a higher sensitivity to ROS, a reduced expression and activity of extracellular laccases and peroxidases associated with a reduced virulence on rice (Guo et al., 2010). H_2O_2 accumulated around the appressorium in the deletion mutant but not in the wild type fungus and inhibition of the rice NADPH oxidases with diphenyleneiodonium (DPI) restored hyphal growth and virulence of the mutant (Guo et al., 2010). The *MoHYR1* gene of *M. oryzae* encodes a glutathione peroxidase GSHPx domain and was shown to be part of the antioxidative defence in *M. grisea*, since *Δhyr1* deletion mutants were impaired in tolerance to H_2O_2 *in vitro* and *in planta* as well as in virulence (Huang et al., 2011). Confocal imaging using fluorescent reporters was used to characterise the cytosolic glutathione redox potential during spore germination, appressorium formation and infection. Results show

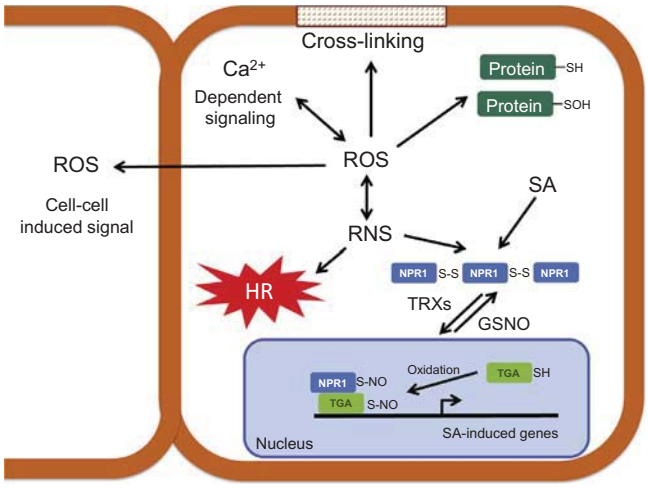

Fig. 1. The multifaceted actions of ROS. The most important ROS-induced mechanisms during plant–microbe interactions are shown. Peroxidase- and ROS-induced cross-linking of cell wall components is part of the defence mechanisms not only against microbes but also insects. ROS can modify multiple biological processes by active post-translational modification (PTM) that includes sulfenylation of cysteine residues (SOH). A well-characterised pathogen-triggered PTM that is dependent on ROS and reactive nitrogen species (RNS), is the reduction and resulting interaction of the SA-induced protein NPR1 and TGA transcription factors. The equilibrium of this induction is regulated by the action of the enzymes S-nitrosoglutathione (GSNO) and thioredoxins (TRXs). Additionally, a defence-induced programmed cell death (PCD), named hypersensitive response (HR) is induced and regulated by the complex crosstalk between ROS and RNS. Finally, ROS can modify other multiple signalling pathways and cell to cell responses induced by different biotic and abiotic stimuli, by the oxidation-dependent regulation of transcription factors and by the co-regulation and co-induction of the secondary messenger Ca^{2+}.

that *M. grisea* is endowed with solid antioxidative defences even during reduced penetration of the fungus in resistant hosts and ROS produced by the host are unlikely to be a direct toxic barrier for the fungus (Samalova et al., 2014). YAP-1 of *Ustilago maydis* is a homolog of the yeast AP-1-like protein that regulates the response to oxidative stress. Deletion mutants of *U. maydis* lacking a functional *YAP-1* gene exhibit a decreased virulence. This is associated with an increase in H_2O_2 detected at the hyphal tips of the penetrating deletion mutant. No accumulation of H_2O_2 was observed around the tips of penetrating hyphae of the wild type fungus. Treating the host plant with DPI restores the virulence of the deletion mutant (Molina and Kahmann, 2007). These studies exemplify how detoxification of host-derived ROS is essential for fungal virulence and pathogenesis.

But ROS can also be sensed by fungal pathogens and act as developmental signals for the differentiation of infection structures (Heller and Tudzynski, 2011). Recent studies succeeded in monitoring the intracellular redox status in *B. cinerea* by expressing a redox-sensitive green fluorescent protein (roGFP) as a biosensor for the redox status in the fungus. This elegant approach showed the importance of intracellular redox differences between infecting hyphae or in appressorial structures during fungal invasion (Heller et al., 2012). Similar observations were made in *M. grisea* lines where glutathione and ROS production where determined by live cell imaging using Grx1-roGFP2 and fluorescent markers (Samalova et al., 2014).

Rather than direct antimicrobial molecules, ROS are more likely cofactors in redox reactions playing various roles in plant defences (Torres, 2010). For instance, ROS have been characterised as primary signalling molecules, regulating multiple physiological processes during plant growth and development (De Tullio, 2010). Interestingly, evolutionary considerations based on the NADPH gene family suggest that mechanisms to detoxify ROS were acquired before the plants used ROS as signalling molecules (Mittler et al., 2011).

The reasons that make ROS important signalling regulators are: (i) fast control over the production and scavenge in individual cells, allowing a dynamic control of ROS levels; (ii) ROS can accumulate in different subcellular organelles, resulting in an efficient intracellular control; (iii) ROS-induced signalling is rapidly propagated from the origin of the stimuli to the rest of the cells; (iv) the chemical nature of ROS allows them to interact and modify different targets (reviewed in Mittler et al., 2011). Remarkably, plants exploit this advantageous versatility of ROS when interacting with the environment and during biotic interactions (Scheler et al., 2013). Here we describe the most relevant roles of ROS during plant–pathogen interactions (Fig. 1).

3.1. Modification of the cell wall

The reinforcement of the cell wall at the site of interaction with the pathogen is an important pathogen-induced defence response. It is mediated by *de novo* pathogenesis-related (PR) protein synthesis including the class III plant peroxidases. Peroxidases mediate ROS-dependent cross-linking of components of the cell wall including glycoproteins, lignin and suberin (Almagro et al., 2009). ROS-mediated cell wall modifications are also involved in the defence of plants against insects. Cell wall modifications mediated by ROS also take part during the interaction of wheat (*Triticum aestivum*) or rice (*Oryza sativa*) with larvae of the Hessian fly (*Mayetiola destructor*) (Liu et al., 2010).

3.2. Signal transduction pathways

ROS regulate different plant hormone signalling pathways, plant–biotic interactions and developmental processes by redox-

dependent regulation of transcription factors (Barna et al., 2012). There is evidence that indicates the direct induction of defence responses mediated by ROS (Torres, 2010). However, under several biotic and abiotic stress conditions the secondary messenger Ca^{2+} is also induced besides ROS accumulation. Since ROS and Ca^{2+} are co-produced and co-regulate each other, the analysis of the regulation of these pathways is complex (Wrzaczek et al., 2013). One of the best characterised defence signalling pathways regulated by oxidation events is the induction of salicylic acid (SA)-dependent responses and attending regulation of two key regulators: the SA receptor NPR1 and TGA transcription factors in *Arabidopsis thaliana* (Liao et al., 2012; Fu and Dong, 2013). Briefly, under non-stress conditions NPR1 is S-nitrosylated at the cysteine-156 by S-nitrosoglutathione (GSNO) and sequestered in the cytoplasm as an oligomer formed by disulphide bounds. Once SA accumulates upon pathogen attack and alters the cellular redox state, this provokes a reduction of the disulphide bonds in the NPR1 protein by the thioredoxins TRX-h3 and TRX-h5 (Tada et al., 2008). Even further, a change in the oxidation state of cysteine residues of TGA transcription factors has been shown to be necessary to promote their interaction with NPR1 in the nucleus (Després et al., 2003). The reduced form of the NPR1 oligomer releases NPR1 monomers that translocate to the nucleus and interact with the oxidised TGA transcription factors, promoting the induction of defence response genes (Tada et al., 2008). Remarkably, ROS accumulation does not always result in an induction of SA-dependent defence responses. During symbiotic interactions in legume roots, ROS production is stronger and lasts longer than during plant–pathogen interactions, but in this case, the expression of PR proteins is reduced (Peleg-Grossman et al., 2012), highlighting the complexity of the ROS-dependent signal transduction.

3.3. Programmed cell death

The programmed cell death (PCD) is an essential mechanism during the growth and development of several organisms including plants. PCD is a highly genetically controlled and orchestrated process that leads to the degradation of proteins, lipids and DNA, destruction of the plasma membrane and phosphatidylserine externalisation, that ends with the destruction of the cell (Dickman and de Figueiredo, 2013). During the last years, PCD in plants has been extensively characterised and several molecular players have been recently described, including the endoplasmic reticulum (ER), Ca^{2+}, nitric oxide (NO) and ROS (reviewed in Agurla et al., in press; Garcion et al., 2014; van Doorn et al., 2011; Williams et al., 2014). In plants, PCD takes place during leaf senescence, photosynthesis and as part of the innate immune response triggered by plant–pathogen interactions, called hypersensitive response (HR). ROS-induced PCD has been described as a mechanism of photooxidative damage during photosynthesis, since plants under severe light stress show PCD induced by an increase in the level and toxicity of singlet oxygen $^1O_2$ (Triantaphylides et al., 2008). Interestingly, a recent analysis indicates that under non-stress light conditions $^1O_2$ can also play a role as signalling molecule, regulating the PCD pathway and generating microscopic lesions in the leaf (Kim et al., 2012). Concerning plant–pathogen interactions, NO and ROS participate in a coordinated way in regulated HR (Bellin et al., 2012). In agreement with this, recent reports have established that reactive nitrogen species (RNS) and ROS are not only crucial players during HR but actually both participate in a complex crosstalk were they can interact and regulate each other (reviewed in Wang et al., 2013). Nevertheless, depending on the biotic interaction, ROS can interfere with PCD induction and regulation or might not be involved at all (Torres, 2010). This indicates that PCD is under a complex regulation, where ROS have an important role but are not the only player.

3.4. Post-translational regulation

ROS have an important role as modulator of different biological processes such as plant–biotic interactions and development, acting on protein post-translational modifications (PTM). ROS can affect PTMs by at least two mechanisms: H_2O_2 can modify the cysteine residues producing a sulfenic acid (–SOH) by the chemical reaction called sulfenylation (Scheler et al., 2013) and oxidation of methionine residues within a phosphorylation motif can inhibit phosphorylation of neighbouring peptides (Hardin et al., 2009). During the symbiotic interaction between *Medicago truncatula* and *Sinorhizobium meliloti* multiple proteins involved in nitrogen fixation are sulfenylated, highlighting the importance of ROS in the establishment and development of this plant–biotic interaction (Oger et al., 2012). On the other hand, *Triticum aestivum* seeds germinated in the presence of cadmium and other oxidative stressors showed that ROS-induced PTM of proteins are involved in cell cycle progression during root growth, affecting G1/S transition and progression through the S phase (Pena et al., 2012).

4. ROS and resistance to fungal pathogens of leaves

In this part we review some examples illustrating the relevance of ROS in the defence of the plant to various, mostly fungal pathogens. The importance of ROS in the defence of the plant has been tested by various attempts to inhibit their production. An efficient way to block ROS production is difficult to find, since several major sources of ROS exist in plant cells. These include a number of enzyme systems that comprise among others NADPH oxidases, superoxide dismutase, oxalate oxidase, cell wall peroxidases, lipoxygenases and polyamine oxidases (Bolwell, 1999; Shetty et al., 2008; Torres et al., 2013; Yoda et al., 2009; Zimmermann et al., 2006).

NADPH oxidases also termed homologues of mammalian respiratory burst oxidases (RBOHs) are associated with ROS formation during the interaction of plants with pathogens (Suzuki et al., 2011). The cDNAs of 2 RBOHs were described in *Nicotiana benthamiana*: *NbRBOHA* expressed constitutively at low levels and *NbRBOHB* induced by the elicitor INF1 from the oomycete *P. infestans* a pathogen of potato. When these genes were silenced by virus-induced gene silencing the suppressed plants show a reduction in ROS accumulation and INF1-induced cell death, along with a loss in resistance to *P. infestans* (Asai et al., 2008; Yoshioka et al., 2003).

Among the 10 sequences encoding RBOHs in *A. thaliana*, *AtRboh D* and *F* have been identified as crucial for ROS formation in leaves and B and C in seed and root respectively (Marino et al., 2012). A decreased ROS production in response to infection with avirulent *Pseudomonas syringae* pv *tomato* DC3000 and *Hyaloperonospora parasitica* was observed in double mutants of *AtRboh D* and *F* (Torres et al., 2002). But rather than generating ROS that act directly in the activation of defences, ROS produced by AtRboh D and F might limit the spread of cell death around bacterial infection sites (Torres et al., 2005). This was shown by studies with *lsd1* (*lesion simulating disease 1*) mutants that form spontaneous lesions that remain localised; when AtRbohD/F are suppressed in *lsd1* mutants as in the triple mutants *lsd1/atRbohD/atRbohF*, the plants develop spreading lesions. In fact, mutants of RbohD are similarly susceptible to a virulent strain of *P. syringae* whereas mutants of RbohF display a slight increase in susceptibility compared to controls (Chaouch et al., 2012).

In potato a Ca^{2+}-dependent protein kinase (StCDPK5) was found to phosphorylate a plasma membrane RBOH leading to an oxidative burst. Transgenic potato expressing a constitutively active form of StCDPK5 under a pathogen-inducible promoter allowed

determining the implication of ROS in the defence of potato to pathogens using a gain-of-function approach. Inoculation of such plants with virulent isolates of *P. infestans* induced ROS and increased resistance to the pathogen. However, transgenic plants were more susceptible to the necrotrophic pathogen *Alternaria solani*. Thus, RBOH-dependent ROS participate in the defence to hemibiotrophic pathogens, but help the necrotrophic pathogen in the colonisation of the tissue (Kobayashi et al., 2012). In agreement with these data, *NbRbohB*-silenced *N. benthamiana* plants develop smaller lesions after inoculation with *B. cinerea* (Asai and Yoshioka, 2009). However, Rboh-mediated ROS accumulation is not strictly correlated with disease susceptibility towards *B. cinerea*. The treatment of Arabidopsis leaves with oligogalacturonides (OGs) elicits an AtRbohD-dependent oxidative burst and protects Arabidopsis plants from subsequent attack by *B. cinerea*. Despite the loss of the OG-elicited ROS-burst, *atrbohD* T-DNA mutants still exhibit an induction of defence genes and an increased resistance towards *B. cinerea* after OG-treatment (Galletti et al., 2008). A recent study suggests nitric oxide (NO) to act as an upstream regulator of the OG-elicited oxidative burst mediated by AtRbohD (Rasul et al., 2012). A link between disease resistance, NO and ROS was also indicated by analysing different Arabidopsis ecotypes after inoculation with *Sclerotinia sclerotiorum*. Resistant ecotypes showed a higher expression of *AtRbohD* and *F* as well as an earlier accumulation of NO and H_2O_2 when compared to susceptible ecotypes during interaction with *S. sclerotiorum* (Perchepied et al., 2010). Adding another layer of complexity to the interplay between NO and ROS, AtRbohD function was shown to be negatively regulated by NO-dependent S-nitrosylation during hypersensitive response (Yun et al., 2011).

The dependence of an oxidative burst on cell wall peroxidases was initially reported in carrot suspension cells (Bach et al., 1993). ROS formation dependent on an apoplastic peroxidase was later confirmed in other plants (bean, Arabidopsis, pepper, lettuce, cotton) (Bestwick et al., 1998; Bindschedler et al., 2006; Bolwell, 1999; Choi et al., 2007; Martinez et al., 1998). The relevance of apoplastic peroxidase was explored in *A. thaliana* expressing an antisense construct of the heme-containing cell-wall-bound class III peroxidase *FBP1* of French bean. In such antisense plants the DPI-insensitive oxidative burst was decreased in response to cell wall preparations of *Fusarium oxysporum*. The antisense plants displayed enhanced susceptibility to a broad range of fungal and bacterial pathogens and showed a decrease in expression of mRNAs coding for peroxidase AtPCa (PRX33) and AtPCb (PRX34) (Bindschedler et al., 2006). When Arabidopsis tissue culture lines generated from *FBP1* antisense plants are treated with ROS inhibitors (azide and DPI) about half of the MAMP-induced H_2O_2 can be accounted for by a peroxidase-generated reaction while the rest is likely to depend on NADPH oxidases and other sources. The expression of MAMP-elicited genes including *MYB51*, *CYP79B2*, and *CYP81F2* and the two cysteine-rich defence-related peptides *PDF2.2* and *PDF2.3* are decreased in the antisense cell lines (O'Brien et al., 2012b). The importance of peroxidase-mediated ROS formation was studied in Arabidopsis T-DNA insertion lines impaired in the expression of the *PRX33* or *PRX34* mRNAs. Mature leaves of such T-DNA knockdown lines respond to MAMPs (Flg22 and Elf26) with reduced ROS formation and callose deposition and a decreased induction of MAMP-activated genes. Finally, the *PRX33* T-DNA knockdown line is more susceptible to *P. syringae* than wild-type plants, supporting a role for peroxidase-mediated oxidative burst in MAMP-mediated plant defence (Daudi et al., 2012).

In Arabidopsis, besides AtRboh the NADP-malic enzyme (NADP-ME) was recently shown to be associated with the formation of ROS and ROS-dependent defences. Inoculation with the hemibiotrophic fungal pathogen *Colletotrichum higginsianum* or with PAMPs (flagellin, chitin) increased the activity of NADP-ME and its transcript levels. In the loss-of-function T-DNA mutant *nadp-me2*, ROS and the formation of callose are decreased in response to PAMPs and an increased susceptibility towards *C. higginsianum* was observed providing experimental support for a role of NADP-ME and associated ROS in plant defence (Voll et al., 2012).

Several necrotrophic pathogens produce oxalic acid (OA) during infection and OA can dampen the elicitor-triggered ROS production (Cessna et al., 2000). An interesting series of studies have tested the importance of OA as a pathogenicity factor and have yielded clues on the relevance of ROS during an interaction with necrotrophic fungal pathogens (for a summary see Walz et al., 2008a). Transgenic tomato expressing a wheat oxalate oxidase displayed oxalate oxidase activity and reduced symptoms after inoculation with *B. cinerea* (Walz et al., 2008b). Overexpression in tobacco of an oxalate decarboxylase from the basidiomycete *Trametes versicolor* that converts OA into CO_2 and formate, leads to a strong ROS accumulation and delayed colonisation of *S. sclerotiorum* (Walz et al., 2008a). Arabidopsis overexpressing the oxalate decarboxylase gene of *T. versicolor* show a faster ROS accumulation after inoculation with *B. cinerea* and a decrease in lesion size compared to controls (L'Haridon et al., 2011). Thus, when necrotrophic pathogens are exposed to an oxidative burst during the initial phase of infection their invasion is weakened. Paradoxically, OA can also induce ROS formation and attending plant cell death at a later stage of the infection that benefits a necrotrophic pathogen. A study by Williams et al. (2011) nicely demonstrates how *Sclerotinia* mediates the redox environment in the host using real-time redox sensing by GFP, histological staining and reverse fungal genetics. At an early phase, *Sclerotinia* creates a reducing environment by secreting OA that prevents the ROS-induced localised defences. This allows an early establishment of the pathogen that at a later stage will exploit host ROS pathways and plant cell death to its own advantage and successful colonisation. A similar role for OA during the later phase of colonisation was highlighted in the interaction of tobacco with *Moniliophthora perniciosa*, the hemibiotrophic causal agent of witches broom disease (da Silva et al., 2011).

ROS are also part of the reactions that are activated when plants undergo priming for defences. Priming of the expression of genes associated with cell wall lignification and of the activity of ROS forming enzymes is induced during systemic resistance in cucumber against *Colletotrichum orbiculare* by acibenzolar-S-methyl treatments (Deepak et al., 2006). Treatments with the PAMP chitosan or with the fungus *Plectosphaerella cucumerina* both induce callose and ROS formation in Arabidopsis plants. When plants are pretreated with the priming agent β-aminobutyric acid (BABA), plants display resistance to the fungus. BABA treatment is also associated with a faster and stronger callose and ROS formation. The priming effect of BABA affects the homeostasis of ROS both by activating the expression and respectively the repression of ROS biosynthetic and scavenging enzymes. This highlights the importance of an oxidised cellular status for activation of defences in primed plants (Pastor et al., 2013). In grapevine suspension cells, BABA primes elicitation of ROS and expression of the *RbohD* gene. In leaves of grapevine, BABA primes for a stronger ROS production in response to the downy mildew agent *Plasmopara viticola* that correlated with an increased resistance to this pathogen. Primed ROS formation and BABA-induced resistance was blocked by DPI (an inhibitor of NADPH oxidoreductases) suggesting that NADPH oxidase-dependent ROS production is crucial to the effect of BABA in grapevine infected by *P. viticola* (Dubreuil-Maurizi et al., 2010).

5. ROS and resistance to fungal pathogens of roots

Although ROS synthesis and function in foliar plant diseases have been described extensively, little is known about the implica-

tion of ROS in defence reactions of the root. The chitin-elicited production of ROS and of H_2O_2 in particular has been demonstrated in roots of *M. truncatula* and Arabidopsis (Kim et al., 2006; Nars et al., 2013; Plancot et al., 2013). Using an elicitor from *Fusarium oxysporum*, increased levels of superoxide and H_2O_2 were also detected in the border-like cells of Arabidopsis and flax roots (Plancot et al., 2013).

The impact of actively growing *F. oxysporum* on ROS levels was demonstrated when elevated amounts of ROS and nitric oxide were visualised in Arabidopsis roots following inoculation (Gupta et al., 2013). Similarly, the generation of H_2O_2 was detected in cotton roots after infection with *Verticillium dahliae* (Xie et al., 2013). In a study investigating H_2O_2 generation upon *V. dahliae* infection, H_2O_2 production in tomato plants carrying the *Ve* resistance gene preceded that observed in a susceptible variety (Gayoso et al., 2010). Transgenic cotton plants expressing a fungal endochitinase gene are more resistant towards *Rhizoctonia solani* and accumulated ROS faster than the wild type after inoculation with the pathogen (Kumar et al., 2009). These data demonstrate that root invasion by a pathogen is accompanied by increased ROS accumulation and suggest that ROS levels are associated to disease resistance.

Our understanding of the ROS-generating processes during root–pathogen interaction is still rudimentary. Bai et al. (2013) detected a reduced expression of an *Rboh* homolog from banana following inoculation with *F. oxysporum* in a resistant cultivar, whereas roots of a susceptible cultivar showed increased *Rboh* transcript levels (Bai et al., 2013). An independent transcriptional analysis of banana roots undergoing compatible interaction with *F. oxysporum* found the induction of an *RbohD* homolog (Park et al., 2012). When expression of the 10 *AtRboh* homologs was compared in *F. oxysporum*-inoculated Arabidopsis roots, *AtRbohD* revealed the most pronounced induction while *AtRboh A*, *B* and *F* were slightly increased. Interestingly, this study also demonstrates that an *atrbohD* insertion mutant is more resistant to *F. oxysporum*, while *atrbohF* plants develop more severe disease symptoms than the wild type (Zhu et al., 2013). Transcriptional analyses of *F. oxysporum*-inoculated banana and Arabidopsis plants also detected an upregulation of several peroxidase genes, but a direct link between peroxidase activity and disease development has yet to be established (Li et al., 2013; Zhu et al., 2013).

The family of germin-like proteins (GLP) have been associated with H_2O_2 accumulation in infected plants (Christensen et al., 2004; Schweizer et al., 1999). GLPs include enzymes with oxalate oxidase (OxO) or superoxide dismutase (SOD) activities leading to H_2O_2 production (Zimmermann et al., 2006). Expression of the *BvGLP-1* gene of sugar beet in Arabidopsis increased the H_2O_2 content in the transgenic plants and conferred resistance to *V. longisporum* and *R. solani* (Knecht et al., 2010). The authors of this study proposed that H_2O_2 produced by *BvGLP-1* may function as a signal activating plant defence responses since the transgenic plants exhibited enhanced transcript levels of both the SA-dependent *PR-1* and *PR-2* genes and the JA/ET-dependent *PR-3*, *PR-4* and *PDF1.2* genes (Knecht et al., 2010). Catalase peroxidase is one of the most prominent upregulated proteins observed when *V. longisporum* senses xylem sap of rapeseed. Reducing the expression of these proteins in *V. longisporum* using RNAi-mediated gene silencing increased the sensitivity of the fungus to ROS and affected the performance of the fungus during the late phases of the disease (Singh et al., 2011). This provides an indirect evidence for the importance of ROS in the defence of rapeseed to *V. longisporum*.

6. Conclusions

ROS are increasingly recognised as important molecules participating in various processes ranging from development to responses of plants to stress. This review focussed on the involvement of ROS in the interaction between plants and pathogenic fungi. Interestingly, ROS can act as developmental signals for the differentiation of infection structures in fungal pathogens. Besides, while ROS are known for their direct antimicrobial role against pathogens they are more likely cofactors in redox reactions playing various roles in plant defences. To this point one of the difficulties remains the localisation of the relevant ROS species, their levels and their dynamics in various tissues and at the cellular level. Our knowledge is limited by a suboptimal temporal and spatial resolution describing ROS kinetics during local and systemic responses. A major challenge in this field will be the development of methods that allow unambiguous detection and quantification of specific ROS. The parallel analysis of ROS in the apoplast as well as in different subcellular compartments will advance as novel technical developments become available. Another challenge is to fully understand how changing ROS levels translate into specific biological outcomes. Such knowledge will help to elucidate the connections among the diverse signalling elements employed by the cell.

Acknowledgement

The support of the Swiss National Science Foundation to JPM is gratefully acknowledged.

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