Phytochemistry xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Review

Reactive oxygen species and plant resistance to fungal pathogens

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Cell wall
Fungal pathogens
Innate immunity
Signalling
Roots

ABSTRACT

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.

Contents

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 will briefly review the available methods to detect the formation

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http://dx.doi.org/10.1016/j.phytochem.2014.08.027
0031-9422/

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. 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

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