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Review

Cross reactivity between ascorbate peroxidase and phenol (guaiacol) peroxidase

Wouter G. van Doorn[a,*], Saichol Ketsa[b]

[a] Mann Laboratory, Department of Plant Sciences, University of California, Davis, CA 95616, USA
[b] Department of Horticulture, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand

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ABSTRACT

Adverse conditions often induce an increase in active oxygen species (AOS) such as hydrogen peroxide (H_2O_2). H_2O_2 is converted to water, and thus becomes detoxified by enzymes such as ascorbate peroxidase (APX; EC 1.11.1.11). APX activity is estimated by the disappearance rate of ascorbic acid, which becomes oxidized. However, ascorbate is also a substrate of guaiacol peroxidase (POX; EC 1.11.1.7). POX oxidizes phenols (including flavonoids), whereby ascorbate accepts an electron from phenoxyl or flavonoid radicals. Ascorbate becomes thereby converted to the monodehydroascorbate radical, which subsequently can become converted to dehydroascorbate. POX isozymes therefore convert hydrogen peroxide to water and oxidize ascorbate, just as APX does. POX activity is usually estimated by monitoring the formation of tetraguaiacol from guaiacol. This reaction is not specific, as some APX isozymes show rather high activity when using guaiacol or similar phenols as a substrate. It is concluded that APX activity can easily be confounded with POX activity, and vice versa. Proper methods should be used to separate the two enzyme activities.

1. Introduction

Stressful abiotic conditions often induce an increase in the antioxidant defense mechanism in plants. Such stress conditions include exposure to high and low temperatures, drought, high light levels, lack of nutrients and the presence of toxic chemicals (Mittler, 2002; Blokhina et al., 2003; Gechev et al., 2006).

Plants are aerobic organisms, and thus utilize oxygen as a terminal electron acceptor. Highly reactive intermediates, reactive oxygen species (ROS), are produced in this process. Oxygen reduction initially produces superoxide and/or hydroperoxide radicals. The oxidizing power of superoxide and hydroperoxide is potentially dangerous. Superoxide can inactivate metabolic enzymes containing Fe–S clusters. Hydroperoxide is found mainly in acidic cellular environments. It can pass biological membranes and initiate lipid oxidation. The production of superoxide and hydroperoxide is followed by that of hydrogen peroxide. Because of its longer half-life, compared to superoxide and hydroperoxide, hydrogen peroxide can migrate to adjacent compartments. Hydrogen peroxide can inactivate enzymes by oxidizing their thiol groups. The destructive properties of superoxide and hydrogen peroxide are even increased when they interact in the presence of metal ions to form the highly reactive hydroxyl radical. This radical can damage almost anything. Because hydroxyl radicals are so highly reactive, cells do not possess enzymatic mechanisms for their detoxification, thus rely on mechanisms that prevent their formation (detoxification of superoxide and hydrogen peroxide, and sequestering of metal ions). Singlet oxygen is most often produced through a reaction with chlorophyll. It can transfer its energy to other molecules and thereby damage them, e.g., the peroxidation of polyunsaturated fatty acids. Plants have evolved a network of ROS-producing and detoxifying enzymes, which in Arabidopsis involves at least about 300 genes (Gechev et al., 2006).

In order to maintain low levels of ROS the cell has two lines of defense, which are not mutually exclusive. The first is the presence of enzymes that detoxify ROS. Superoxide dismutase (SOD) converts superoxide into hydrogen peroxide (Fig. 1). Catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX) convert hydrogen peroxide to water (Fig. 1). The second line of defense is the presence of endogenous antioxidative chemicals, some of which are the substrate of antioxidative enzymes (ascorbate and glutathione for APX and GPX, respectively) while others can act independent of these enzymes, for example phenols and anthocyanins and other flavonoids (Apel and Hirt, 2004). Singlet oxygen produced in chloroplasts can be detoxified (quenched) by carotenoids (Gechev et al., 2006).

* Corresponding author.
E-mail address: wgvandoorn@ucdavis.edu (W.G. van Doorn).

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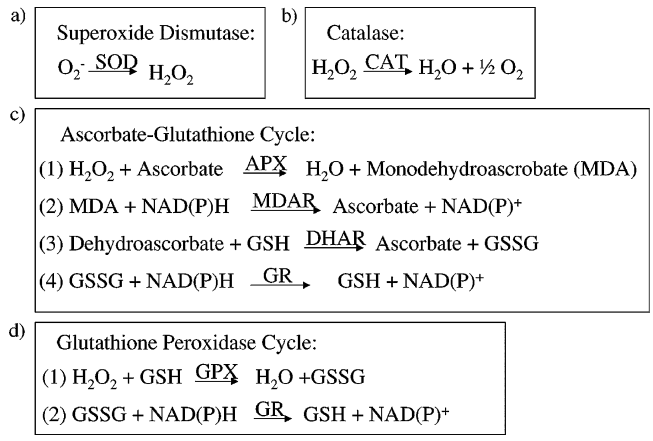

Fig. 1. Production and detoxification of hydrogen peroxide (after Apel and Hirt, 2004).

Peroxidases are grouped into three classes, of which classes I and III are found in plants (Mathé et al., 2010). APX (EC 1.11.1.11) is a class I peroxidase (Gumiero et al., 2010; Singh et al., 2012). Its biological functions have been reviewed (Noctor and Foyer, 1998; Shigeoka et al., 2002; Mittler, 2002; Ishikawa and Shigeoka, 2008; Lazzarotto et al., 2011). APX activity is apparently found in cell walls (Mehlhorn et al., 1996), in the cytosol, and in chloroplasts, mitochondria and peroxisomes (Foyer and Noctor, 2003). APX activity is usually assessed by the rate of disappearance of ascorbic acid.

Class III peroxidases (PODs; EC 1.11.1.7) can also contribute to maintaining low levels of hydrogen peroxide levels (Grace and Logan, 2000; Mathé et al., 2010). Many POD reactions require hydrogen peroxide, which is converted to water, while the substrate is converted into a free radical. Class III peroxidases consist of a large number of proteins, involved in an array of functions such as cell wall cross linking and cell wall loosening, lignin and suberin formation, auxin metabolism, defence against pathogens and insects, and coping with physical stress (Passardi et al., 2005; Cosio and Dunand, 2009; Marjamaa et al., 2009).

Here, we will discuss only one group of PODs, which shows high activity with guaiacol as a substrate. These enzymes will here be called POX. To estimate POX activity it is customary to monitor the formation of tetraguaiacol from guaiacol. Anderson et al. (1995) observed that all major POX isozymes were also able to use *p*-phenylenediamine as a substrate. Additionally, some POX isozymes react with flavonoids, i.e., more complex phenolics. The term POX excludes enzymes using monolignins as lignin building blocks (Ranieri et al., 2001).

Several POX isozymes are found in the vacuole, where no APX is present (Mehlhorn et al., 1996; Yamasaki and Grace, 1998). The vacuole contains phenolics which can act as electron donors in the POX reaction (Yamasaki and Grace, 1998). Other POX isozymes are found in cell walls, in addition to cell wall PODs such as those involved in lignin and suberin formation. Cell wall POXs have been suggested to participate in defence responses (Durner and Klessig, 1995) and have been linked to cell wall separation during abscission (Henry et al., 1974). Still other POX isozymes are found in the cytosol, but in contrast to APX, they are not found in organelles (Asada, 1992; Shigeoka et al., 2002).

This review will discuss the proposition that the common methods to determine APX and POX activity are not specific. This is based on the findings (a) that POX reactions can continuously use ascorbate as the electron acceptor, just as APX, and (b) that some APXs show rather high affinity to guaiacol and similar phenolic substrates.

The topic has a bearing on human health. Antioxidants from food oppose the action of ROS and reactive nitrogen species, which both can cause damage to DNA and other molecules, and can induce diseases. Both ascorbate and flavonoids are important antioxidants in plant-based foods (Halliwell, 1996; Ross and Kasum, 2002; Willcox et al., 2004; Liu, 2004; Tripoli et al., 2005). The rate of their formation and degradation are therefore important for food quality.

2. Role of APX and POX in resistance to oxidative stress

Adverse environmental conditions, such as heat or cold or drought, often induce an increase in ROS concentrations in plant cells. The crucial role of APX in countering the increase in ROS has been reviewed in several papers (e.g., Suzuki and Mittler, 2006; Ishikawa and Shigeoka, 2008; Foyer and Noctor, 2011; Gallie, 2013). Enhanced expression of *APX* genes and increased APX activity has been found in response to environmental stress (Graham and Foyer, 1998). APX-antisense transgenic tobacco was highly susceptible to oxidative stress (Örva and Ellis, 1997) and knockout of a cytosolic APX in rice inhibited growth during drought, cold and salt stresss (Zhang et al., 2013). Overexpression of an *Arabidopsis* peroxisomal APX in tobacco increased resistance to oxidative stress (Wang et al., 1999), while overexpressing a barley APX in *Arabidopsis* increased its resistance to high temperature and salt (Shi et al., 2001). Badawi et al. (2004) reported that over-expression of APX in tobacco chloroplasts enhanced the tolerance to water deficit and salt stress, while overexpression of cytosolic APX in tomato conferred tolerance to chilling and salt stress (Wang et al., 2005), both also related to improved ROS metabolism. Overexpression of an APX in rice plants also increased chilling tolerance (Sato et al., 2011). In non-transgenic plants or plant parts, the alleviation of chilling injury was also associated, almost without exception, with an increase in APX activity (see examples below).

Less is known about the role of POX in counteracting stress-induced increases in ROS concentrations. Here some examples will be discussed in which POX activity was increased, related to a decrease in ROS concentrations. Some other examples will also be noted in which either no increase in POX activity was detected, or even a decrease was found, but increased resistance to oxidative stress was nevertheless observed.

Increased POX activity has been observed during exposure to low temperature of whole plants such as coffee (Queiroz et al., 1998), cucumber (Lee and Lee, 2000; Kang and Saltveit, 2002; Xu et al., 2008), maize and rice (Kang and Saltveit, 2002). Other types of abiotic stress also resulted in an increase in POX activity, for example drought (Zhang and Kirkham, 1996), hypoxia (Bai et al., 2010), exposure to NaCl (de Azevedo Neto et al., 2006), cadmium ions (Metwally et al., 2003) or ozone (Kronfuss et al., 1996). Noctor and Foyer (1998) opined that POXs are among the major antioxidative enzymes in plant cells.

Arabidopsis plants overexpressing a tobacco *POX* gene exhibited increased tolerance to aluminium toxicity and exhibited less aluminium-induced oxidative stress (Ezaki et al., 2000). Water-stress damage in cucumber (*Cucumis sativus* L.) seedlings was alleviated after treatment with spermidine. This was accompanied by an increase of POX activity and, to a lesser degree, a reduction of SOD and catalase activities. Hydrogen peroxide and superoxide radical contents were reduced in the water-stressed plants, following the treatment (Kubiś, 2008).

Populus cathayana plants held at 4 °C showed chilling stress, whereby female plants were considerably more negatively affected than male ones. The activities of glutathione reductase and POX significantly decreased at low temperature in females but not in males, thus POX activity was higher in males (Zhang et al., 2011).