Journal of Plant Physiology 221 (2018) 101–106

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Short communication

Selective responses of class III plant peroxidase isoforms to environmentally relevant UV-B doses

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ARTICLE INFO

Keywords:
Peroxidase
Substrate dependence
Leaf
Tobacco
Ultraviolet light

ABSTRACT

Efficient hydrogen peroxide detoxification is an essential aspect of plant defence against a large variety of stressors. Among others, class III peroxidase (POD, EC 1.11.1.7) enzymes provide this function. Previous studies have shown that PODs are present in several isoforms and have in general low substrate specificities. The aim of our work was to study how various assays based on using various substrates reflect differences in peroxidase activities of tobacco leaves due to either developmental or environmental factors. The former factor was studied comparing fully developed leaves of the 3rd and 5th nodes; and the latter was achieved using plants acclimated to low doses of supplementary UV-B (280–315 nm) in growth chambers. To investigate the above, POD activities were measured using three different, commonly used chromophore substrates: ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), guaiacol (2-methoxyphenol), OPD (*o*-phenylenediamine) and a fourth substrate, the secondary metabolite quercetin. All substrates registered a UV-B induced increase in leaf peroxidases as compared to untreated controls, although to different extents. However, age-related differences between upper and lower leaves were only detectable when either ABTS or quercetin were used as substrates. Additionally, native PAGE separation of POD isoforms followed by visualisation using one of the substrates showed that leaf acclimation to supplementary UV-B is realized via a selective activation of POD isoforms.

1. Introduction

Class III plant peroxidases (PODs) are present as large multigene families in all plants (Cosio and Dunand, 2009). PODs are primarily localized in cell walls and vacuoles (Passardi et al., 2005), but according to Aiamla-or et al. (2014) these are also present in chloroplasts. During POD catalyzed oxidative reactions the enzyme converts hydrogen peroxide (H_2O_2) to H_2O while a substrate is being oxidised (Hiner et al., 2002). PODs are well-known for their wide substrate specificity that also allows the use of various synthetic chromophores as electron donors to assess POD activities of leaf extracts. This low substrate specificity does not necessarily grant equal substrate oxidation rates by various POD isozymes with putative functional specialization (Cosio and Dunand, 2009). Experiments with Mediterranean thyme (*Thymbra spicata* L.) showed that substrate preference of POD enzymes strongly depends on the applied assay's pH or temperature as well (Doğan et al., 2007). Upregulation of POD activities was observed as a general plant stress response to salt stress (Stevens et al., 1978), high and low temperature (Gulen and Eris 2004; Kim et al., 2012) or UV-B (Majer et al., 2014). POD isoenzymes can respond differently to stress effects. Ranieri et al. (2001) found that iron deficiency caused a rearrangement of POD isoforms in sunflower leaves. Sreenivasulu et al. (1999) found an acidic POD isoform in foxtail millet (*Setaria italic* L.) to be important in tolerating salinity stress. Increased activities of specific POD isoenzymes in response to extremely high doses of UV-B were reported in sunflower cotyledons (Yannarelli et al., 2006) and buckwheat (*Fagopyrum esculentum*) seedlings (Jovanovic et al., 2006). However, responses of POD isoforms to environmentally relevant low UV-B doses have not been studied so far.

In order to study how changes in POD activities and isoenzymes are assessed by various assays using different substrates we chose *Nicotiana tabacum* as model plant. The aspect of our investigation was dual: (i) we considered leaf age as an internal factor potentially affecting leaf POD (Takahama et al., 1999) (ii) UV-B radiation has been applied as an external factor enhancing POD activities (Majer et al., 2014; Czégény

Abbreviations: ABTS, 2,2′ azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); -C, control plants exposed to PAR only; F_v/F_m, maximum quantum efficiency of PS II; L-, leaves from the 3rd node of the plants, lower leaves; HRP, horseradish peroxidase; OPD, *o*-phenylenediamine; POD, total peroxidase; Y(NO), non-regulated non-photochemical quenching of PS II; Y(NPQ), regulated non-photochemical quenching of PS II; U-, leaves from the 5th node of the plants, upper leaves; -UV, plants exposed to PAR and supplemental UV radiation; ϕPSII, light acclimated effective quantum yield of *PsII*

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https://doi.org/10.1016/j.jplph.2017.12.010
Received 14 June 2017; Received in revised form 14 November 2017; Accepted 8 December 2017
Available online 16 December 2017
0176-1617/

et al., 2016). UV-B is a key regulator of plant growth and development (Jansen, 2002; Jenkins, 2014) but it is also a potential stressor capable of both increasing leaf H_2O_2 concentrations and photo-converting H_2O_2 to hydroxyl radicals (Czégény et al., 2014). Consequently, PODs have central role in plant UV-acclimation (Majer et al., 2014; Czégény et al., 2016). In addition to three artificial substrates commonly applied in plant POD assays: 2-methoxyphenol (guaiacol), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and *o*-phenylenediamine (OPD) we also included quercetin, a leaf secondary metabolite in the comparative studies presented here. Tobacco leaves synthesize glycosylated quercetins (Li et al., 2003) and *in vitro* experiments demonstrated that these forms also act as POD substrates in addition to the aglycone (Yamasaki et al., 1997). Leaf responses to the applied UV-B treatment and potential age-related differences in physiological status were monitored using non-invasive pigment and photosynthesis measurements.

2. Materials and methods

2.1. Plant material and UV-treatment

Tobacco (*Nicotiana tabacum* L. cv. Petite Havanna) plants were grown on standard garden soil in growth chambers (Fitotron, SGC 120 Plant Growth Chamber, Weiss Technik UK, Loughborough, UK) under 150 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) using long day conditions (16/8 h, 25/20 °C). Four weeks after emergence, plants were divided into two groups. One group (UV plants) was exposed to supplemental UV-B radiation for 5 days, while the other group (control plants) was kept under the same conditions as earlier. After 5 days, two fully-developed leaves from each plant (from 3rd and 5th nodes, marked as lower and upper leaves) were used for the analyses below. Difference in the distance of upper and lower leaves from light sources was approximately 12 cm. Due to inner reflexions in the growth chamber, this difference was not reflected in either PAR or UV irradiances as measured using a FLAME-S-UV-VIS spectroradiometer (Ocean Optics Inc., Dunedin, FL, USA).

Supplemental UV radiation was applied from Q-Panel UVB-313EL tubes (Q-Lab Ltd., Bolton, UK) through a cellulose diacetate filter (Courtaulds Chemicals, Derby, UK) between 10 AM and 2 PM daily. The UV spectrum was centred at 318 nm and the irradiation corresponded to 7.7 kJ m^{-2} d^{-1} biologically effective dose (BED) as calculated using the Biological Spectral Weighting Function developed by Flint and Caldwell (2003). The applied BED was approximately the same as the ambient daily UV-B measured at latitude N46° during summer earlier (Bassman et al., 2001).

2.2. Non-invasive pigment and photosynthesis measurements

Leaf flavonoid content was characterized by the adaxial side flavonoid index measured with a Dualex Scientific™ optical sensor (Force-A, Orsay, France). Following this measurement, plants were kept in darkness for 30 min before photosynthetic activities were characterized by chlorophyll fluorescence using the MAXI-version of the Imaging PAM (Heinz Walz GmbH, Effeltrich, Germany). First minimum and maximum fluorescence yields (F_0 and F_m, respectively) were determined before and after a saturating pulse applied to the dark adapted leaf, and the maximal effective Photosystem (PS) II quantum yield was calculated as $F_v/F_m = (F_m - F_0)/F_m$. Following this, the leaf was illuminated with blue actinic light corresponding to 55 μmol m^{-2} s^{-1} PAR applied for 4 min and fluorescence yields F' and F'$_m$ were measured before and after a saturating pulse. PS II photochemistry was characterized by the yield, $\phi PSII = (F'_m - F')/F'_m$; and non-photochemical energy dissipation processes were characterized by the non-regulated yield $Y(NO) = F'/F_m$ and the regulated yield $Y(NPQ) = F'/F'_m - Y(NO)$ (Klughammer and Schreiber, 2008).

2.3. Peroxidase (POD) activity measurements

Following the above non-invasive measurements, leaves were detached, frozen in liquid N_2 and stored at −80 °C until further use. Frozen leaves were powdered in liquid N_2 using a pestle and mortar and extracted into ice cold sodium-phosphate buffer (50 mM, pH 7.0) containing 1 mM EDTA. Leaf homogenates were centrifuged (24.400 × *g* for 30 min at 4 °C, Hettich Rotina 380 R, Andreas Hettich GmbH, Tuttlingen, Germany) and supernatants were kept at −20 °C until use.

POD (EC 1.11.1.7) activities were measured using four different assays, all measured in 50 mM phosphate-citrate buffer (pH 5.0). Reaction mixtures contained 400 μM H_2O_2 and one of the following substrates: (i) 183 mM ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), (ii) 2.06 mM guaiacol (2-methoxyphenol), (iii) 50 mM OPD (*o*-phenylenediamine) or (iv) 2.96 mM quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one). One mL reactions mixtures contained 6–20 μL leaf extracts and peroxidase activities were quantified via measuring substrate oxidation for 3 min using a spectrophotometer (Shimadzu UV1800, Shimadzu Corp., Kyoto, Japan). The oxidation of ABTS was followed at 651 nm (Childs and Bardsley, 1975). Guaiacol or OPD oxidation was measured at 450 nm (Fielding and Hall, 1978; Fornera and Walde, 2010). Quercetin oxidation was measured indirectly, via the oxidation of 14 mM ascorbate at 295 nm. Ascorbate is oxidised while restoring quercetin that is oxidised as peroxidase substrate (Yamasaki et al., 1997). Results were corrected for the non-enzymatic H_2O_2 scavenging by ascorbate and quercetin contained in the assay that was less than 13% of enzymatic activities measured in leaf samples.

Protein contents of leaf extracts were determined using the standard Bradford assay (Bradford, 1976) and POD activities were expressed as units mg^{-1} protein using the following molar extinction coefficients of substrates: A_{651} (ABTS) = 10.22 mM^{-1} cm^{-1}, A_{450} (guaiacol) = 5.98 mM^{-1} cm^{-1}, A_{450} (OPD) = 15.75 mM^{-1} cm^{-1}, A_{295} (ascorbate) = 0.1 mM^{-1} cm^{-1} that we obtained from absorption spectra.

It is important to note that for the sake of comparison all substrates were used at pH 5.0. This is the standard condition for all assays, with the exception of the guaiacol method that is usually used at pH 7.0 (Fielding and Hall, 1978). While guaiacol oxidation in the POD reaction is as efficient at pH 5.0 as at 7.0 (Doğan et al., 2007), POD units measured with OPD at pH 7.0 were only 20% of values measured at 5.0, and both ABTS and quercetin were unable to act as POD substrates at pH 7.0 (data not shown).

2.4. Native PAGE

To determine various possible UV-B activated POD isoenzymes, samples were first separated on 12% SDS free native tris-glycine gels (PAGEr® Gold Precast Gels Lonza). After separation gels were rinsed in distilled water, and then were soaked in 4 mM H_2O_2 for 10 min. Finally staining procedures were carried at room temperature. The staining solution contained one of the following chromophore substrates: 183 mM ABTS, 2.06 mM guaiacol or 50 mM OPD in 50 mM phosphate-citrate buffer (pH 5.0). Greyish-blue (ABTS), brown (guaiacol) or orange (OPD) activity POD bands and marker (No. 26619, PageRuler Plus Prestained Protein Ladder, Thermo Scientific) bands appeared on a colourless background. For comparison, the standard calibrating enzyme of POD assays, horseradish peroxidase (HRP) was also separated on the gels.

2.5. Statistics

For each measurement, two leaves were collected from four treated and four control plants. POD activity measurements were repeated three times. Results are shown as means ± standard deviations. Differences between datasets were evaluated using unpaired Student's *t*-