J. Plant Biochem. Biotechnol. (October–December 2014) 23(4):440–443
DOI 10.1007/s13562-014-0272-8

SHORT COMMUNICATION

Responses of sweet potato peroxidases to sodium nitroprusside-mediated nitric oxide

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Received: 26 December 2013 / Accepted: 28 April 2014 / Published online: 7 May 2014

Abstract To ascertain the response of sweetpotato peroxidases (PODs) to nitric oxide (NO), we treated the leaves of sweet potato with the NO generator sodium nitroprusside (SNP) and the NO scavenger carboxyl-PTIO (cPTIO). Exogenous application of more than 5 mM SNP caused damage to sweetpotato leaves at 24 h after treatment. The accumulation of NO in leaves was positively correlated with the SNP dose. The specific activity of PODs in sweet potato leaves was markedly increased by treatment with greater than 1 mM SNP for 24 h, whereas POD activity and accumulated NO content decreased to low levels by treatment with cPTIO. Expression analysis of POD genes in response to treatment with SNP and cPTIO revealed that major stress-inducible acidic genes, such as *swpa1*, *swpa2*, *swpa3*, and *swpa4*, were specifically regulated. These results indicate that increased NO levels in sweet potato leaves are closely linked to an improved defense capability mediated by stress-inducible PODs.

Keywords Nitric oxide · Peroxidase · Sweet potato · Sodium nitroprusside · Carboxyl-PTIO

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Abbreviations

cPIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
POD	Peroxidase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SNP	Sodium nitroprusside

Nitric oxide (NO) is a relatively stable paramagnetic gaseous free-radical and key signaling molecule involved in many physiological processes, where it functions as a synchronizing chemical messenger involved in cytotoxicity and programmed cell death during pathogen attack in plants (Besson-Bard et al. 2008). NO is also implicated in the response to some abiotic stresses, which has been reported in plants (Corpas et al. 2008). NO and most of the reactive nitrogen species (RNS), such as $NO^{\bullet}$, NO^{+}, NO^{-}, $NO^{\bullet}_{2}$, peroxynitrite ($ONOO^{-}$), *S*-nitrosothiols (RSNOs), and *S*-nitrosoglutathione (GSNO), are major signaling molecules in plants. They can be synthesized during stress responses at the same time as generation of reactive oxygen species (ROS) (Durner and Klessig 1999 At the transcriptional level, a microarray study obtained from NO donor-treated *Arabidopsis* cells indicates that NO modulates the expression of several defense-related genes, including genes encoding pathogenesis-related and secondary metabolism-related proteins (Parani et al. 2004).

Class III plant peroxidases (PODs, EC 1.11.1.7) play a key role in these responses, and are involved in a broad range of physiological processes throughout the plant life cycle, including the metabolism of ROS and RNS (Almagro et al. 2009). Although there is correlative evidence for the involvement of POD activity with response to NO accumulation (Huang et al. 2002), the regulation of different POD

isoenzymes during exposure to NO is not currently understood. We previously isolated 13 POD genes from sweet potato and characterized their expression levels under environmental conditions (Kim et al. 2007, 2010). However, the response of sweet potato POD genes to NO has not been characterized in detail. In the current study, we analyzed POD-specific activity and the expression of the 13 POD genes in leaves of sweet potato in response to treatment with exogenous NO donors sodium nitroprusside (SNP) and scavengers 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO).

For SNP treatment, the third leaves from the top were detached from each plant and transferred to Petri dishes containing 50 mL of each solution at increasing concentrations for 24 h. SNP affected sweet potato leaves, and higher concentrations induced greater effects. Treatments with greater than 5 mM SNP for 24 h caused severe cellular damage of leaves. Cell death resulted from treatment with 50 mM SNP for 24 h (Fig. 1a). NO content was analyzed according to the method of Tossi et al. 2011). The accumulated NO content of sweet potato leaves increased in response to treatment with greater than 0.5 mM SNP for 24 h (Fig. 1b). After treatment with 1–50 mM SNP for 24 h, POD activity in leaves increased by 1.5−2-fold compared with that in untreated leaves (Fig. 1c). We also performed a time-course analysis of NO content and POD activity in sweet potato leaves treated with 1 or 5 mM SNP for 24 h. The results show that NO was strongly increased after treatment with 1 or 5 mM SNP for 6 h (Fig. 1d). Fig. 1e shows that there is no significant difference between POD activity after treatment with 1 or 5 mM SNP for 2 h and that of untreated controls. By contrast, POD activity increased 1.8 and 2.5-fold after SNP treatment compared with untreated conditions for 24 h. Even though the NO levels was strongly increased by 5 mM SNP treatments, the POD activity did not significantly produce by 5 mM SNP treatment. When considering the internal NO levels in the sweetpotato leaves, the difference of POD activity between 1 mM and 5 mM SNP became even less remarkable. It is possible that the similar level of a POD activity under over 1 mM SNP treatments may be threshold effects of internal NO levels in the sweeetpotato leaves.

To investigate the NO-specific responses of sweet potato PODs, we analyzed NO content, POD activity and expression in response to treatment with SNP and NO scavenger cPTIO.

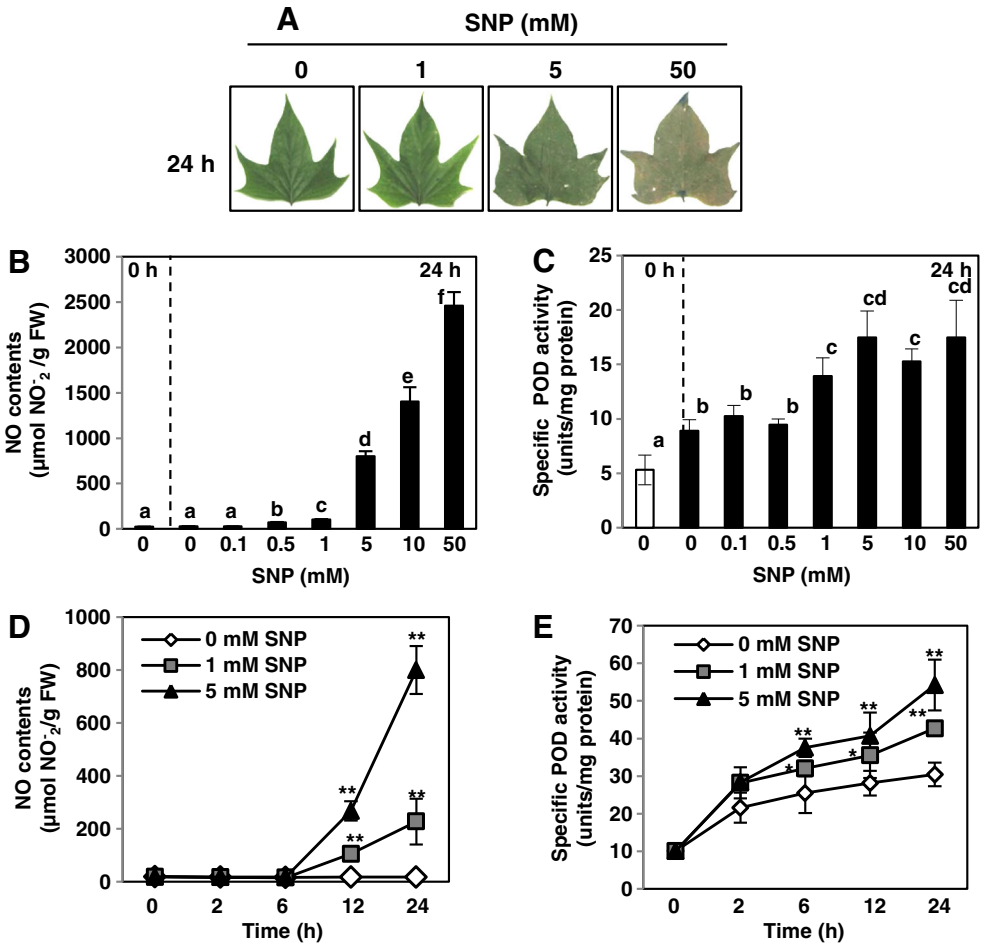

Fig. 1 Effect of NO in sweetpotato leaves treated with SNP for 24 h. (A) Visible damage of sweetpotato leaves. (B) Accumulation of internal NO content. (C) Change of POD activity. (D) Accumulation of internal NO content during a time-course study. (E) Changes of POD activity during a time-course study. Bars denoted with the same letter are not significantly different according to Duncan's multiple range test (P=0.05) and one-way ANOVA with LSD post-hoc test (*P<0.05; **P<0.01)

To determine if NO production correlates with POD activity, the patterns of POD activities were analyzed during treatment with SNP and cPTIO (Figs. 2a and b). To prevent NO accumulation, sweet potato leaves were incubated in 0.1 mM cPTIO for 2 h before SNP treatments. The results showed that POD activity in leaves treated with cPTIO correlated with the changes in NO accumulation in response to 1 or 5 mM SNP. This suggests that cPTIO-induced inhibition of NO affected POD activity in sweet potato leaves. We also investigated the responses of 13 POD genes to increasing concentrations of SNP in leaves of sweet potato. Quantitative RT-PCR was performed in a fluorometric thermal cycler (DNA Engine Opticon 2, MJ Research, USA) using EverGreen fluorescent dye. The experimental control comparisons of repeated samples were assessed using CT values between the three replications. Linear data were normalized to the mean CT of α-tubulin as a reference gene. The expression levels of sweet potato POD genes were analyzed by quantitative RT-PCR

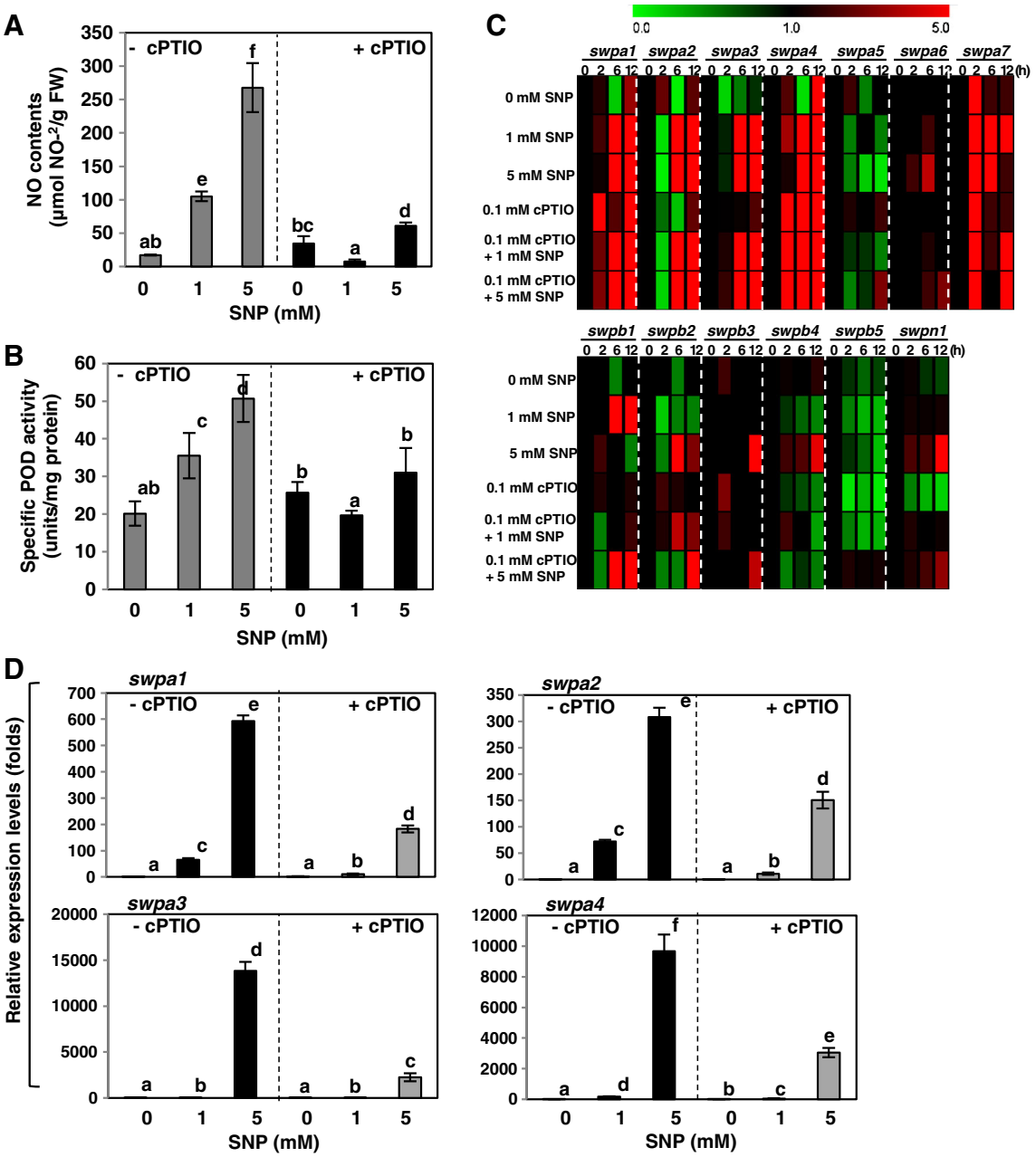

Fig. 2 Effect of SNP and cPTIO in sweetpotato leaves for 12 h. (A) Changes in NO content in sweetpotato leaves. (B) Change of POD activity. (C) Comparison of expression levels of 13 POD genes in leaves of sweetpotato plants leaves. The heatmap using MULTIEXPERIMENT VIEWER VERSION 4.9 (MEV4.9) displays normalized values for each gene transcript for each sample. (D) Expression patterns of stress-inducible POD genes in sweetpotato leaves by quantitative RT-PCR analysis. Bars denoted with the same letter are not significantly different (P=0.05) according to Duncan's multiple range test

using the gene-specific primers reported in the previous study (Kim et al. 2010). The 13 POD genes exhibited different expression patterns in response to increasing concentrations of SNP (Fig. 2c). Previously we reported the expression profiles of 13 POD genes in sweet potato in response to air pollutants and UV radiation (Kim et al. 2007). The four acidic POD genes, *swpa1*, *swpa2*, *swpa3*, and *swpa4* specifically involved in the defense against oxidative stress were induced under multiple stress conditions. In this study, the four major stress-inducible acidic POD genes, *swpa1*, *swpa2*, *swpa3*, and *swpa4*, were also strongly expressed in response to SNP treatment. To investigate the NO-specific response of these four POD genes, we analyzed the gene expression patterns in response to treatment with SNP and NO scavenger (Fig. 2d). After treatment with 5 mM SNP for 24 h, expression levels of four POD genes strongly induced by 592 folds (*swpa1*), 308 folds (*swpa2*), 13,807 folds (*swpa3*) and 9,662-folds (*swpa4*) compared with that in untreated leaves. SNP-induced expression of the four major POD genes decreased in response to cPTIO treatment for 12 h. Expression patterns of four POD genes were similar to the changes in POD activities under SNP and cPTIO treatment conditions. This result suggests that changes in NO levels, induced by treatment with SNP and cPTIO, specifically regulate the four major stress-inducible acidic POD genes in sweet potato leaves. NO functions as a messenger molecule that is involved in signaling regulation, triggering tolerance against various abiotic and biotic stresses, thus it is the key molecule controlling the activation of various defense-related genes (Durner et al. 1999). Therefore, it was suggested that NO may play a major role in the signaling cascade leading POD gene expression under various stress conditions.

The four NO-induced POD genes identified in the current study are potential candidate genes for the development of transgenic plants to study the function of POD in terms of NO regulation. Among the four NO-induced POD genes identified, overexpression of *swpa4* in transgenic tobacco plants was previously reported (Kim et al. 2008). Recently, *swpa4*-overexpressing transgenic plants also showed increased NO levels under normal and pathogen-infected conditions compared with control plants (unpublished data). Therefore, our results suggest that the expression of *swpa4* may function as a positive defense signal in the ROS or NO-related stress response signaling pathway.

In conclusion, we evaluated changes in POD enzymatic activity and the expression profiles of POD genes in leaves of sweet potato treated with the NO donor SNP and the NO scavenger cPTIO. Our results showed that the activity and gene expression of four stress-inducible PODs in sweet potato leaves were regulated by accumulated NO levels in response to SNP and cPTIO. The four POD genes induced specifically by NO represent potential tools for the generation of transgenic plants for functional studies of POD in terms of NO regulation.

Acknowledgments This work was supported by grants from the National Center for GM Crops (PJ008097) and the Systems & Synthetic Agrobiotech Center (PJ008119), Biogreen21 Project for Next Generation, Rural Development Administration, Korea, and the KRIBB initiative program.

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