

Research article

Differential expression of 10 sweetpotato peroxidases in response to sulfur dioxide, ozone, and ultraviolet radiation

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Abstract

Secretory class III plant peroxidase (POD, EC 1.11.1.7) is believed to function in diverse physiological processes, including responses to various environmental stresses. To understand the function of each POD in terms of air pollutants and UV radiation, changes in POD activity and expression of 10 POD genes isolated from cell cultures of sweetpotato (*Ipomoea batatas*) were investigated in the leaves of sweetpotato after treatment with sulfur dioxide (SO₂ 500 ppb, 8 h/day for 5 days), ozone (O₃ 200 ppb, 8 h/day for 6 days), and ultraviolet radiation (UV-B 0.6 mW m⁻² for 24 h, UV-C 0.16 mW m⁻² for 24 h). All treatments significantly reduced the PSII photosynthetic efficiency (F_v/F_m). POD-specific activities (units/mg protein) were increased in leaves treated with SO₂ and O₃ by 5.2- and 7.1-fold, respectively, compared to control leaves. UV-B and UV-C also increased POD activities by 3.0- and 2.4-fold, respectively. As determined by RT-PCR analysis, 10 POD genes showed differential expression patterns upon treatment with air pollutants and UV radiation. Among the POD genes, *swpa1*, *swpa2*, and *swpa4* were strongly induced following each of the treatments. Interestingly, basic POD genes (*swpb1*, *swpb2*, and *swpb3*) were highly expressed following SO₂ treatment only, whereas neutral *swpn1* was highly induced following O₃ treatment only. These results indicated that some specific POD isoenzymes might be specifically involved in the defense mechanism against oxidative stress induced by air pollutants and UV radiation in sweetpotato plants.

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1. Introduction

Air is one of the most important natural environments affecting living organisms. However, the atmosphere is among the first components of the environment to experience environmental pollution [30], and air pollutants may cause significant

health and environmental problems. Despite the long history and significant consequences of this problem, the development of effective legal remedies is a relatively recent undertaking. As a result of human activities, concentrations of SO₂ (sulfur dioxide) and O₃ (ozone) in the lower atmosphere have been considerably increased in industrialized parts of the world. SO₂ is a phytotoxic pollutant that reacts with other pollutants to produce other phytotoxins in the troposphere, including O₃ and acidic substances [5]. Furthermore, the release of chlorofluorocarbons into the atmosphere may cause the depletion of stratospheric O₃, and may increase the transmittance of ultraviolet-B (UV-B) radiation to the surface of the Earth [27].

Abbreviations: CAT, catalase; F_v/F_m , current photochemical capacity of PSII; DAT, days after treatment; HAT, hour after treatment; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; UV, ultraviolet.

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Increased intensities of UV-B could affect plant growth and enhance photochemical reactions that generate some phytotoxic pollutants.

Oxidative stress derived from reactive oxygen species (ROS) is one of the major factors affecting plant productivity when plants are exposed to various environmental stresses. Atmospheric concentrations of SO₂, O₃, and UV radiation may reach levels that are toxic to plants. Plants usually respond by triggering various defense mechanisms; one such response that has been invoked in various environmental stress conditions is the antioxidant defense system. The plant antioxidant system consists of protective enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidases (PODs), as well as of low-molecular weight antioxidants such as ascorbate, glutathione, α -tocopherol, and carotenoids [1,21]. SOD converts superoxide radicals (O₂⁻) into hydrogen peroxide (H₂O₂). CAT dismutates H₂O₂ into water and oxygen, whereas PODs reduce H₂O₂ to water using various substrates as electron donors. In the presence of O₂⁻ and H₂O₂, trace amounts of transition metals can give rise to the highly toxic hydroxyl radical (OH⁻). Therefore, rapid detoxification of both O₂⁻ and H₂O₂ is essential for preventing oxidative damage. Among the antioxidant enzymes, the induction of specific isoenzymes of the PODs in many plants has been recognized as being among the biomarkers of various environmental stresses [6].

Secretory class III peroxidase (POD, EC 1.11.1.7) catalyzes the reduction of H₂O₂ by taking electrons to donor molecules such as phenolic compounds, lignin precursors, auxin, or secondary metabolites [23], and has been implicated in a broad range of physiological processes such as lignification, suberization, auxin metabolism, cross-linking of cell wall compounds, and defense against biotic and abiotic stresses [10,23]. However, the complexity of the physiological processes in which POD isoenzymes are involved makes it difficult to understand the specific function of each of these enzymes. Therefore, the specific functions of these POD genes in terms of plant growth and adaptation to environmental stress remain to be determined. PODs in plants have been suggested to be involved in defense mechanisms against air pollutants and UV radiation [25]. Despite the correlative evidence for the involvement of POD activity with resistance to air pollutants and UV radiation, the regulation of PODs during air pollutant and UV radiation treatments on an isoenzyme basis remains poorly understood.

In previous studies, we established an efficient production system for PODs in suspension cultures of sweetpotato (*Ipomoea batatas* L.) [16,18]. Ten POD cDNAs, six anionic (*swpa1*, *swpa2*, *swpa3*, *swpa4*, *swpa5*, and *swpa6*), three basic (*swpb1*, *swpb2*, and *swpb3*) and one neutral (*swpn1*) cDNA, were isolated from cell cultures of sweetpotato, and their expression levels were characterized in order to determine the physiological functions of each POD in response to various environmental stresses such as wounding, chilling, stress-related chemicals, and pathogen infection [11,12,14,15,22]. However, the responses of sweetpotato POD genes to air pollutants and UV radiation have not yet been characterized in detail. Only four POD genes, *swpa1*, *swpa2*, *swpa3*, and *swpn1*, were investigated upon exposure to O₃ [14]. In this

study, we analyzed the responses of 10 POD genes in leaves of sweetpotato treated with SO₂, O₃, and UV radiation. In addition, PSII photosynthetic activity and changes in POD activity were also investigated after treatment with air pollutants and UV radiation.

2. Results

2.1. Effects of air pollutants and UV treatment on PSII photosynthetic efficiency

The photosynthetic efficiency in sweetpotato leaves was significantly affected by treatment with air pollutants such as SO₂, O₃, and UV radiation (Fig. 1). Under treatment conditions in this study, the photosynthetic efficiency was found to slowly respond to treatment with SO₂ and O₃, whereas that was rapidly decreased upon exposure to UV radiation. Inhibition of the photosynthetic activity in the leaves of sweetpotato appeared from 2 days and 1 day after treatment (DAT) with SO₂ and O₃, respectively (Fig. 1A and B). However, the leaves of sweetpotato remained green at 2 DAT with air pollutants (data not shown), while severe chlorosis was observed in the sweetpotato leaves at 5 DAT and 6 DAT with SO₂ and O₃, respectively. Sweetpotato plants treated with SO₂ and O₃ showed approximately 27.5% and 28.4% reduced photosynthetic activity compared to non-treated control plants at 5 and 6 days, respectively. Photosynthetic activity in sweetpotato leaves treated with UV-B and UV-C was significantly reduced from 3 h after treatment (HAT), and at 24 HAT, the photosynthetic activity was finally reduced by approximately 55.9% and 76.4%, respectively, compared to that of the control plants (Fig. 1C and D).

2.2. Changes of POD activity in stress-treated sweetpotato leaves

Specific POD activity (units/mg protein) was measured in leaves of sweetpotato treated with air pollutants and UV radiation after the final treatment with each stress. POD activity in non-treated (NT) control plants showed a similar level, ranging from 2.12 to 3.45, even though we investigated the enzyme activities in independent experiment for each individual stress. All treatments significantly increased the POD activity depending on the sources of the stress (Fig. 2). POD activity in sweetpotato leaves treated with SO₂ and O₃ was dramatically increased by approximately 5.2- and 7.1-fold, respectively, compared to NT control plants. UV-B and UV-C treatments also increased the POD activity by approximately 3.0- and 2.4-fold, respectively, compared to NT control plants. The POD isoenzyme patterns shown by native gel assays following treatment with each stress reflected the increased POD activity (data not shown). However, the staining patterns of PODs differed in terms of SO₂, O₃, and UV radiation, indicating that several POD isoenzymes are specifically involved in overcoming the oxidative stresses derived from air pollutants and UV radiation. The POD isoenzyme patterns following exposure to UV-B and UV-C radiation were very similar.

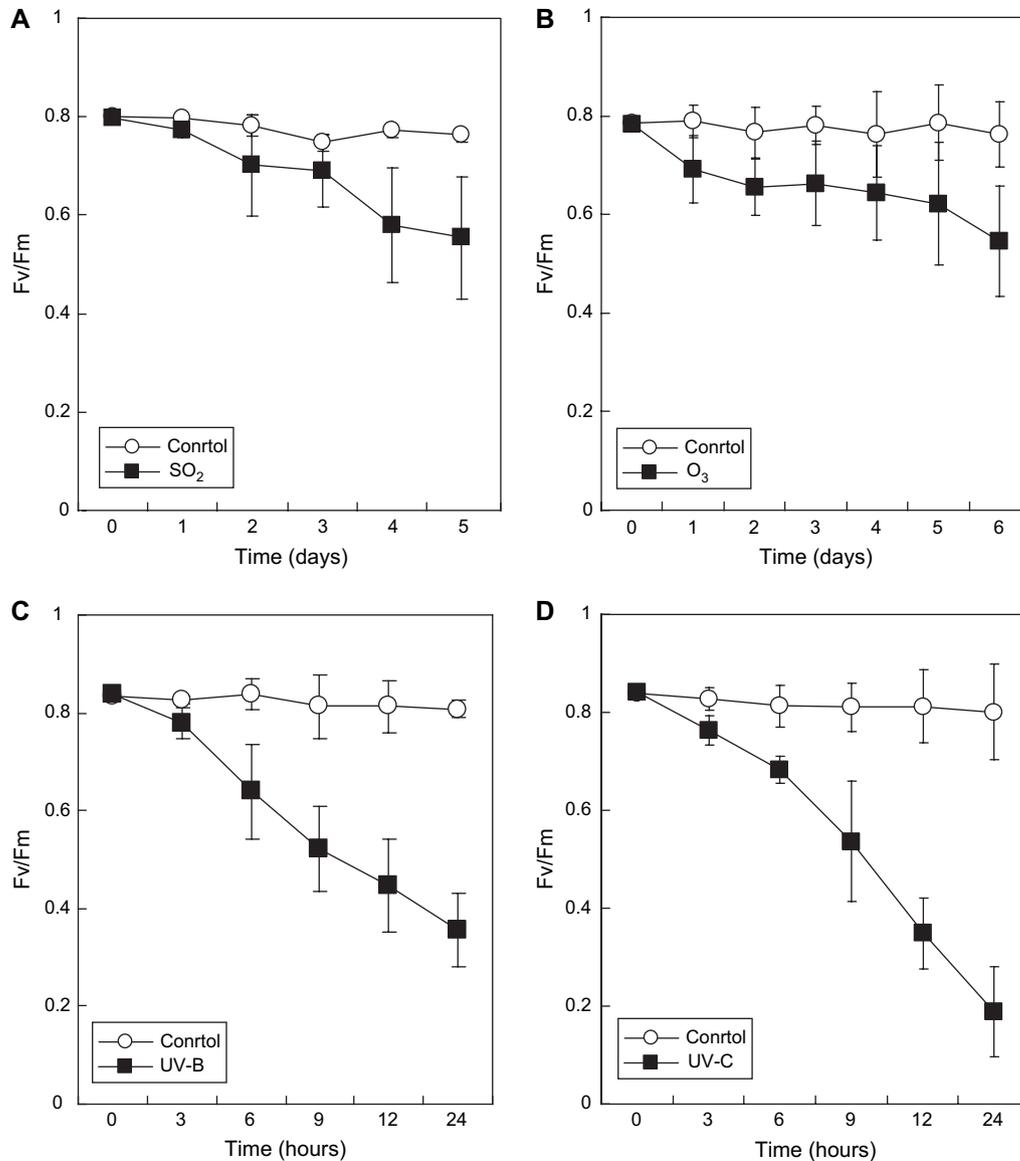


Fig. 1. Changes in photosynthetic efficiency (F_v/F_m) in leaves of sweetpotato plants exposed to SO_2 , O_3 , and UV radiation. The extent of plant damage was quantified by F_v/F_m , which is a measure of the PSII photosynthetic rate. Photosynthetic activity in leaves of sweetpotato exposed to (A) 0 and 500 ppb SO_2 for 8 h per day over 5 days; (B) 0 and 200 ppb O_3 for 8 h per day over 6 days; (C) 0 and 0.6 mW cm^{-2} UV-B for 24 h; and (D) 0 and 0.16 mW cm^{-2} UV-C for 24 h. Data are shown as means \pm SD of three independent measurements.

2.3. Differential expression of 10 sweetpotato POD genes

Using RT-PCR analysis, the responses of 10 POD genes isolated from cell cultures of sweetpotato were investigated in sweetpotato leaves that were treated with air pollutants and UV radiation. Total RNA was extracted from plant leaves after final treatment of each stress, which occurred at 5 days (SO_2), 6 days (O_3), and 1 day (UV). Fig. 3 shows the gene expression and relative expression values (T/NT) of the treatment group (T) to the non-treatment group (NT) following stress treatment. The 10 POD genes showed diverse expression patterns upon treatment with air pollutants such as SO_2 , O_3 , and UV radiation. Ten POD genes were weakly expressed in non-treatment (NT) samples. Thus T/NT values were calculated on the basis

of each non-treated control. SO_2 treatment increased the expression levels of several POD genes, including four anionic PODs (*swpa1*, *swpa2*, *swpa3*, and *swpa4*) and three basic PODs (*swpb1*, *swpb2*, and *swpb3*), by more than two times compared to the NT control. The expression levels of four PODs, *swpa1*, *swpa2*, *swpa4*, and *swpn1*, were increased to two times higher than those of the NT group upon treatment with O_3 . In the case of UV treatment, four anionic POD genes, *swpa1*, *swpa2*, *swpa3*, and *swpa4*, were highly induced by treatment with UV-B and UV-C radiation, but other genes were not expressed following UV radiation treatment. Interestingly, three anionic PODs, *swpa1*, *swpa2*, and *swpa4*, were induced following all of the treatments. Three basic PODs, *swpb1*, *swpb2*, and *swpb3*, were expressed only upon treatment with SO_2 , whereas *swpn1* responded only to O_3 treatment.

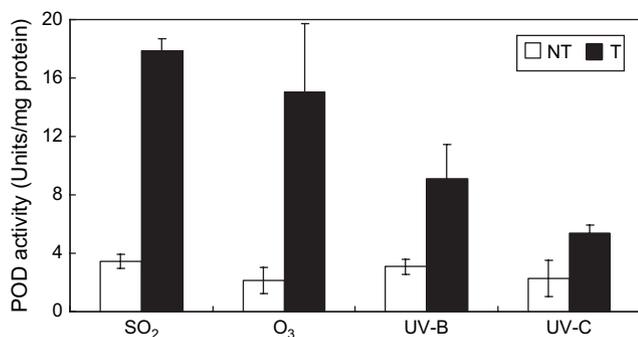


Fig. 2. Changes in peroxidase (POD) activity (units per mg protein) in leaves of sweetpotato plants exposed to air pollutants and UV radiation. SO₂ at 500 ppb for 8 h per day and O₃ at 200 ppb for 8 h per day were treated for 5 days and 6 days, respectively. UV-B at 0.6 mW cm⁻² and UV-C at 0.16 mW cm⁻² were treated for 12 h. Data are shown as means ± SD of three independent measurements.

These results indicate that some specific POD genes are involved in defense in relation to air pollutant- and UV radiation-mediated oxidative stresses in sweetpotato plants.

3. Discussion

Physiological studies have demonstrated that a complex array of processes is employed by plants to adapt to abiotic stress conditions. Plant PODs play important roles in terms of plant defense mechanisms. External stimuli first contact the plant cell walls, and external effects are mitigated by POD reactions such as the removal of ROS.

Various researchers have reported decreased photosynthetic activity following exposure to air pollutants and UV radiation

[8,28]. Sweetpotato plants treated with SO₂ showed a 27.5% reduction of photosynthetic activity at 5 DAT (Fig. 1A). SO₂ produces many kinds of ROS, including O₂⁻, H₂O₂, and OH⁻, when it converts HSO₃⁻ or SO₃²⁻ to SO₄²⁻ in plant cells [28]. Therefore, it has been suggested that the destruction of PSII in sweetpotato plants is caused by the presence of excess ROS upon treatment with SO₂. POD activity was increased by 5.2-fold at 5 DAT after treatment with SO₂ (Fig. 2). It could be expected that the enhanced activity of POD was correlated with SO₂ treatment, which would suggest that POD plays an oxidative stress-protective role against the accumulation of ROS upon treatment with SO₂ in sweetpotato.

In the case of O₃ treatment, the photosynthetic activity of sweetpotato was decreased by 28.4% at 6 DAT (Fig. 1B). O₃ enters plant tissues through open stomata, where it reacts with components of the cell wall and plasma membranes to form various ROS [13,17]. The physiological disturbances caused by O₃ result in a low photosynthetic efficiency. POD activities in sweetpotato were strongly induced, showing a 7.1-fold increase at 6 DAT upon treatment with O₃ (Fig. 2). This finding suggests that an antioxidant enzyme such as POD in the apoplast may play an important role in defending against the generation of ROS caused by O₃ treatment. Along similar lines, the induction of POD activity was implicated by SO₂ and O₃ treatment in various plants such as *Arabidopsis* and *Cassia siamea* [24,25].

At 24 HAT, sweetpotato plants also showed reductions in photosynthetic activity of about 55.9% and 76.4% caused by UV-B and UV-C radiation, respectively, compared to control plants (Fig. 1C and D). The primary target of UV radiation is the reaction center of PSII, the most sensitive protein complex of the photosynthetic electron transfer chain. Damage to

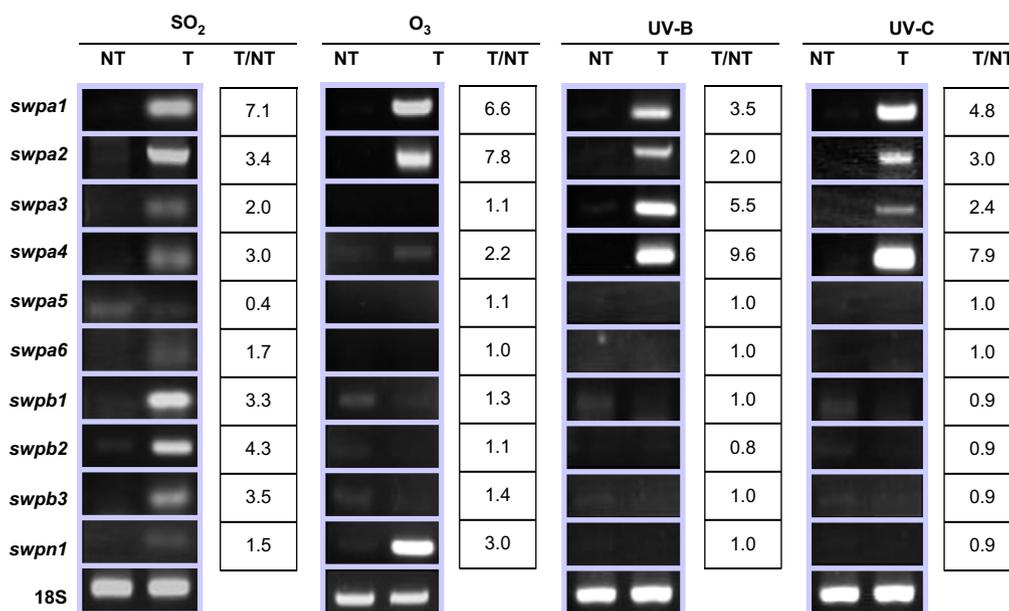


Fig. 3. RT-PCR analysis of the expression of 10 peroxidase genes in leaves of sweetpotato plants exposed to air pollutants and UV radiation. Total RNA was extracted from plant leaves at 5 days after SO₂ treatment, at 6 days after O₃ treatment, and at 1 day after UV (UV-B and UV-C) treatment by the Trizol method (Invitrogen, USA). After reverse transcription (performed as described by the kit manufacturer), PCRs were performed using primers that were specific for each gene. 18S RNA was used as a control for equal loading. Reaction products (20 μl) were analyzed by gel electrophoresis.

this complex results in a loss of photosynthetic capacity and degradation of reaction center proteins, D1 and D2 [2]. Increased levels of ROS such as O_2^- and H_2O_2 are detected simultaneously with the photo-inhibition induced by UV irradiation. Sweetpotato plants treated with UV-B and UV-C showed increased levels of POD activity of approximately 3.0- and 2.4-fold, respectively, at 24 HAT (Fig. 2). UV radiation generates H_2O_2 for the synthesis of secondary metabolites such as lignin. POD activity and lignin contents increase during exposure to UV radiation in quinoa seedlings [9]. POD activity in plants can increase in response to a variety of stresses, including air pollutants and UV radiation, and POD activities have been suggested to be involved in cell wall cross-linking, diferulic bonds, extensin, lignification, suberization, and in responses to abiotic and biotic stresses [23]. In addition, POD catalyzes the polymerization of naturally occurring phenolic compounds in response to chemical stresses such as heavy metal stress [19].

Our previous data on 10 POD genes from cell cultures of sweetpotato showed mostly induced expression profiles in response to various abiotic stresses and pathogenic infection [11,12,14,15,22]. When we observed restricted responses using limited dosage and time of exposure to air pollutants and UV radiation in this study, some POD genes, such as *swpa1*, *swpa2*, and *swpa4*, exhibited a strong response regardless of stress sources, while some genes showed a stress source-specific response. Considering these characteristics, we can classify the 10 POD genes in response to air pollutants and UV radiation on the basis of gene expression levels (Fig. 4A). We counted POD as a positive response gene showing more than two times higher relative expression values (T/NT) of treatment (T) to non-treatment (NT) after exposure to stress. As a result, three anionic PODs, *swpa1*, *swpa2*, and *swpa4*, were induced by each of the treatments. These three genes were similarly expressed following treatment with various environmental stresses [11,12,14,15,22]. Three basic PODs, *swpb1*, *swpb2*, and *swpb3*, were expressed only upon treatment with SO_2 , whereas *swpn1* responded to only O_3 treatment. *Swpa3* responded to both SO_2 and UV radiation treatment. The expression levels of *swpa5* and *swpa6* were not significantly changed by any of the treatments. Among the 10 genes, seven are SO_2 -inducible, four are O_3 -inducible, and four are UV radiation-inducible. These results suggest that the 10 POD genes were induced via complex signaling pathways. Interestingly, the responses of the 10 POD genes in this study are well-correlated with the phylogenetic tree of the POD genes on the basis of amino acid sequences (Fig. 4B). Phylogenetic tree on 10 POD genes in this study can be divided into four groups. Four anionic POD genes such as *swpa1*, *swpa2*, *swpa3*, *swpa4* belong to the first group, which are responded to diverse air pollutants. The second is *swpn1* with O_3 -specific response. The third one is two anionic genes such as *swpa5* and *swpa6* with no response to air pollutants. The last one is three basic POD genes such as *swpb1*, *swpb2*, and *swpb3* with SO_2 -specific response. Our data suggest the responses of 10 POD genes in this study are well-correlated with the phylogenetic tree, suggesting that each POD

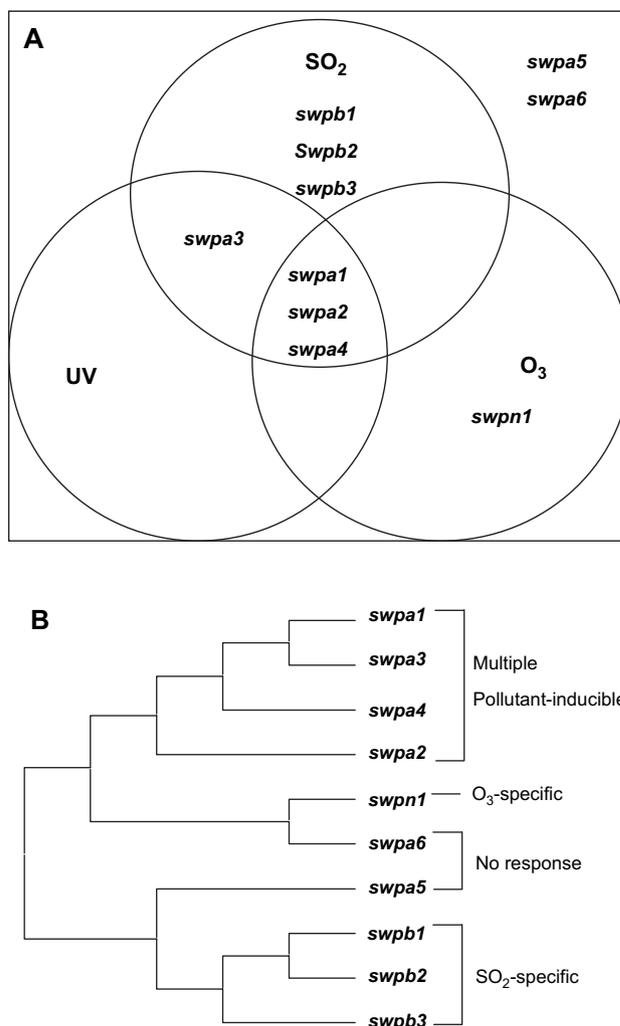


Fig. 4. Classification of sweetpotato POD genes according to their response to air pollutants and UV radiations. Ten POD genes were classified by their responses in the leaves of sweetpotato treated with SO_2 , O_3 and/or UV in Fig. 3. (A) Positive response POD gene indicates that showing more than two times high relative expression value (T/NT) of treatment (T) to non-treatment (NT) after stress treatment. (B) An unrooted peroxidase (POD) tree (dendrogram) showing the evolutionary distance of 10 sweetpotato peroxidases. The tree was constructed from the putative mature proteins using the TreeView program.

gene might be specifically involved in the evolutionary adaptation to each environmental stress.

These diverse or redundant expression patterns indicate an indispensable function of each sweetpotato POD for tolerance to environmental stresses. The functions of individual POD genes may differ from each other, as suggested by the characteristic stress-responsive expression profiles shown in this study. The redundant expression observed after stress treatments indicated the necessity of POD activities for plant self-defense against environmental stresses. Here, we showed the various expression profiles of each POD gene in sweetpotato plants. However, information in this study on the characteristics and roles of sweetpotato POD enzymes is limited.

For a better understanding of the roles of individual POD gene, experiments using transgenic plants with suppression or enhancement of expression of each POD gene are necessary. In addition, studies of transgenic plants will be useful for functional analysis of the POD gene under various stresses. In the previous report, transgenic tobacco plants expressing *swpa1* showed an enhanced tolerance to MV-mediated oxidative stress [29].

In this study, interestingly, the *swpa4* gene was strongly expressed in response to UV-B and UV-C radiation, by 9.6- and 7.8-fold, respectively. In previous studies, the *swpa4* gene was found to be markedly expressed in response to wounding and bacterial infection [12,22]. In addition, the expression level of *swpa4* was increased by abscisic acid, methyl jasmonate, and ROS such as exogenous methyl viologen (MV) and H₂O₂ [22]. Exposure to large amounts of UV radiation causes the expression of stress-associated genes, in part through the activation of pathogen defense and wound-signaling pathways [4,7,20]. Moreover, ROS are generated by the regulation of UV radiation and stress signal molecules such as salicylic acid, ethylene, and jasmonic acid [26]. Therefore, further functional study of the *swpa4* gene will be interesting in terms of UV radiation. Recently, we developed transgenic tobacco plants expressing *swpa4* under the control of a CaMV35S promoter. *Swpa4* transgenic tobacco showed enhanced tolerance to multiple stresses against various abiotic and biotic stresses (unpublished data). In addition, promoter analysis of *swpa4* genomic encoding *swpa4* cDNA is under study in terms of multiple environmental stresses, including heavy metal stress.

4. Materials and methods

4.1. Plant materials and stress treatment

Sweetpotato (*I. batatas* cv. Yulmi) plants were grown in a greenhouse for six weeks, and were exposed to air pollutant treatments. Plants were fumigated with SO₂ at a concentration of 500 ppb for 8 h per day over five days at closed growth chamber, whereas they were fumigated with O₃ at a concentration of 200 ppb for 8 h per day over six days at closed growth chamber. The temperature and relative humidity in the chamber were controlled at 25 °C and 60% relative humidity (RH) with a cooling and humidifying system. For UV radiation treatment, the third leaves from the top were detached from each plant and incubated in Falcon tubes containing 30 ml of sterilized distilled water at 25 °C for 24 h. Plant leaves were exposed to UV-B light (300–315 nm, 0.6 mW cm⁻²) in a growth chamber and UV-C light (240–260 nm, 0.16 mW cm⁻²) at a clean bench.

4.2. Measurement of PSII photosynthetic efficiency

The efficiency of photosynthetic activity in leaves treated with air pollutants and UV radiation was estimated by chlorophyll fluorescence determination of photochemical yield (F_v/F_m), which represented the maximal yield of the photochemical

reaction on photosystem II, using a portable chlorophyll fluorescence meter (Handy PEA, Hansatech, England) after 30 min of dark adaptation. Measurements were performed at room temperature (25 °C) using saturating light flashes (2000 μmol m⁻² s⁻¹).

4.3. Peroxidase assay

The sweetpotato leaves were homogenized on ice with a mortar in 0.1 M potassium phosphate buffer (pH 6). The homogenate was centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was used immediately for POD assays. Protein concentrations were determined according to the Bradford [3] method using Bio-Rad protein assay reagents and bovine serum albumin (BSA) as a standard. POD activities were assayed according to the method described by Kwak et al. [18], using pyrogallol as substrate. The standard assay reaction mixture in a total volume of 3 ml contained enzyme solution (0.1 ml), 0.1 M potassium phosphate buffer (pH 6, 0.32 ml), 5% pyrogallol (0.32 ml, w/v), 0.147 M H₂O₂ (0.16 ml), and H₂O (2.1 ml). The reaction was initiated by the addition of H₂O₂, and the increase in A₄₂₀ nm was recorded for 20 s. One unit of POD activity is defined as that forming 1 mg of purpurogallin from pyrogallol in 20 s at 20 °C (pH 6).

4.4. RT-PCR analysis

Total RNA was extracted from sweetpotato leaves using the Trizol method. For RT-PCR, 1 μg of total RNA was used as a template for reverse transcription with 500 ng of oligo (dT)12–18 primer (Promega, Madison, WI, USA) and the Improm-II™ reverse transcription enzyme (Promega, Madison, WI, USA) for 1 h at 42 °C. PCR amplification reactions were initially incubated at 94 °C for 5 min, followed by 25–35 cycles at 94 °C for 30 s, 42–58 °C (depending on the melting temperature of each gene-specific primer) for 30 s, and 72 °C for 30 s. Reaction products (20 μl) were analyzed by gel electrophoresis. As an internal control of reverse transcription, 18S internal standards (Ambion) were used. Gene-specific primers for PCRs were designed from the 3'UTRs or the region near the translation stop codon of each gene. The *swpa1* primer set (5'-GAGATTGCTTGCTTCACCCGACTA-3', 5'-ATTTT TGGAGCATATCATCAT-3') amplifies a 201-bp product from cDNA coding for *swpa1*; the *swpa2* primer pair (5'-TTAATATTGAAAACCCCTT-3', 5'-CATTTCGACCAACGT TATTAC-3') generates a 361-bp product; the *swpa3* primer set (5'-CATGTTTGCAGCGAGGTG-3', 5'-TGATGAACATA GTAACAACAAC-3') yields a 213-bp product; the *swpn1* primer pair (5'-GGTGACGCGCCGTGAGGAGGTA-3', 5'-CC AAAGGGAGAGGGAAAATCACA-3') amplifies a 183-bp product. The primer sets of the other POD genes were as described in a previous report [22]. The nucleotide sequences of RT-PCR products were identical to those of the corresponding POD cDNAs. RT-PCR band intensity was quantified by Bio-1d software program (VIBER LOURMAT, Germany).

Acknowledgments

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