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Original article

# Differential expression of 10 sweetpotato peroxidase genes in response to bacterial pathogen, *Pectobacterium chrysanthemi*

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**Abstract**

To understand the function of each peroxidase (POD, EC 1.11.1.7) in terms of biotic stress, changes in POD specific activity and expression of 10 POD genes were investigated in four cultivars of sweetpotato (*Ipomoea batatas*) after infection with *Pectobacterium chrysanthemi*. POD specific activity (units mg$^{-1}$ protein) increased from 16 h after inoculation (HAI) in three varieties. POD activities of two cultivars, Shinwhangmi and White Star, reached a maximum level at 24 HAI by about three times compared to mock treatment (MT), and then decreased, whereas those of Zami and Yulmi continuously increased until 36 HAI. Native gel analysis revealed that one POD isoenzyme with a high electrophoretic mobility significantly increased in response to pathogen infection in all cultivars. Additionally, 10 POD genes displayed differential expression patterns upon bacterial infection by northern analysis. Several POD genes such as *swpa2, swpa3, swpa4, swpa5, swpb1* were induced upon bacterial infection, but other genes were not. Particularly, *swpa4* gene was markedly expressed in response to bacterial infection in four different cultivars, suggesting that this gene has a stress-inducible promoter. These results indicate that some specific POD isoenzymes are involved in defense in relation to pathogenesis of *P. chrysanthemi* in sweetpotato plants.
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*Keywords:* *Ipomoea batatas*; Biotic stress; Convolvulaceae; Native gel analysis; Northern analysis; Peroxidase; *Pectobacterium chrysanthemi*

## 1. Introduction

Secretory class III peroxidases (PODs, EC 1.11.1.7) oxidize a vast array of compounds (hydrogen donors) in the presence of $H_2O_2$. PODs are found in a large family of isoenzymes in individual plant species [10,31]. They have been implicated in a broad range of physiological processes such as lignification, suberization, auxin metabolism, cross-linking of cell wall proteins, defense against pathogen attack, salt tolerance and oxidative stress [1,3,8,10,22,24,31,32]. POD activity in plant cells is often modulated by environmental stresses, both abiotic and biotic [3]. But the complexity of the physiological processes in which POD isoenzymes are involved makes it difficult to understand the specific function of each of theses enzymes.

During the last decade, several molecular biological approaches have been developed to isolate, characterize and study the expression of POD genes in plants. So far, POD cDNAs and genes have been isolated by molecular cloning from more than 20 plants including horseradish, arabidopsis and rice [6,9,11,30]. Recently the analysis of the *Arabidopsis* genomic sequence has revealed that there are 73 genes for guaiacol-type PODs in *Arabidopsis thaliana* [29]. The expression profiles of 23 POD genes in various tissues of *A. thaliana* have been investigated by RT-PCR analysis. However, the specific functions of these POD genes in terms of plant growth and adaptation to environment stress remain to be determined. PODs in plants have been suggested to be involved in defense against pathogen infection [19] in addition to abiotic stress. Despite the correlative evidence for the involvement of POD activity with pathogen resistance, the POD regulation during pathogen infection on the isoenzyme

*Abbreviations:* MT, mock treatment; PAGE, polyacrylamide gel electrophoresis; POD, peroxidase.

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basis are largely unknown [5,27], because many POD isoenzymes were simultaneously expressed in plants in response to pathogen infection [4,28].

In previous studies, we established an efficient production system for PODs from suspension cultures of sweetpotato (*Ipomoea batatas*) [17,20]. Ten POD cDNAs, six anionic *swpa1, swpa2, swpa3, swpa4, swpa5* and *swpa6*, three basic *swpb1, swpb2* and *swpb3*, and one neutral *swpn1*, were isolated from cell cultures of sweetpotato and their expression levels were characterized to begin to understand the physiological functions of each POD in response to various abiotic stresses such as wounding, chilling, ozone, and stress-related chemicals [12,14,15,26]. Furthermore, we recently isolated a *SWPA2* genomic clone encoding *swpa2* POD with a strong oxidative stress-inducible promoter [16]. The *SWPA2* promoter was characterized in transgenic tobacco plants and cultured cells in terms of stress responses, and the data obtained suggested that it will be biotechnologically useful for the development of transgenic plants with enhanced tolerance to environmental stress and particularly transgenic cell lines engineered to produce key pharmaceutical proteins [16]. But the response of sweetpotato POD genes to biotic stress was not characterized. In this study, we analyzed the expression patterns of 10 POD genes in four cultivars of sweetpotato infected with *Pectobacterium chrysanthemi*, an important pathogen in sweetpotato cultivation. Changes in POD activity were also investigated after pathogen infection. The results highlight the potential utility of the *swpa4* gene with a strong expression level during pathogen infection for development of transgenic plants with enhanced tolerance to multiple stresses.

## 2. Results and discussion

### 2.1. Change in POD activity during bacterial infection

POD specific activities (units mg$^{-1}$ protein) in four cultivars of sweetpotato were measured in leaf discs infected with *P. chrysanthemi* (Fig. 1). POD activity was somewhat increased from 16 h after inoculation (HAI) in three cultivars; Yulmi, Shinwhangmi and White Star, in response to *P. chrysanthemi* infection. In cultivar Zami, POD activity increased from 20 HAI on pathogen infection, whereas mock treatment (MT) only slightly increased POD activity compared to the untreated control (0 h). The increase of POD activity in MT may be derived from wounding, since most POD genes including sweetpotato respond to wounding. POD activities of two cultivars; Sinwangmi and White Star, reached a maximum level at 24 HAI by about three times compared to MT, and then decreased (Fig. 1C, D), whereas those of Zami and Yulmi continuously increased until 36 HAI (Fig. 1A, B). These results suggested that PODs are involved in defense mechanism in relation to pathogenesis of *P. chrysanthemi* in sweetpotato plants.

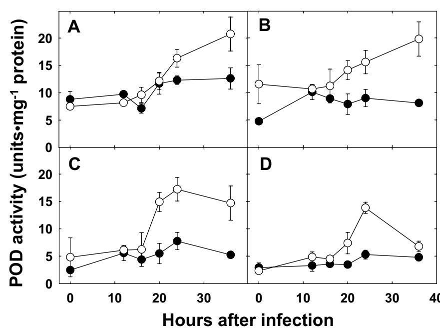


Fig. 1. Changes in POD specific activity (units mg$^{-1}$ protein) in four cultivars of sweetpotato (*I. batatas*) after infection with *P. chrysanthemi*. A, cv. Zami; B, cv. Yulmi; C, cv. Shinwhangmi; D, cv. White Star. (●): Pathogen treatment; (○): MT. Data are mean ± S.E. of three independent replications.

To identify which POD isoenzymes are involved in the increase of POD activity after pathogen infection, the protein samples (12, 24, and 36 HAI) were electrophoresed on native-polyacrylamide gels and POD isoenzymes were stained with benzidine and hydrogen peroxide (Fig. 2). The native gel analysis showed that there were several different bands between MT and pathogen treatment (T) regardless of varieties. Surprisingly, one isoenzyme with the highest electrophoretic mobility (marked with a arrowhead in Fig. 2) was significantly increased in response to pathogen infection, suggesting that this isoenzyme is most likely involved in defense mechanism in relation to pathogenesis of *P. chrysanthemi* in sweetpotato plants. This isoenzyme also slightly increased in MT involved in wounding.

POD activity in plants can increase in response to a variety of stresses including biotic stress, indicating that POD activities have been suggested to be involved in, for example, cell wall biosynthesis by the polymerization of cinnamyl alcohol into lignin [23], in polymerization of extensin [8], in defense against attack by pathogens [19], and in the response to wounding [7,21]. In addition, POD catalyzes polymerization of naturally occurring phenolics to produce a variety of bioactive products, suggesting that POD reaction products

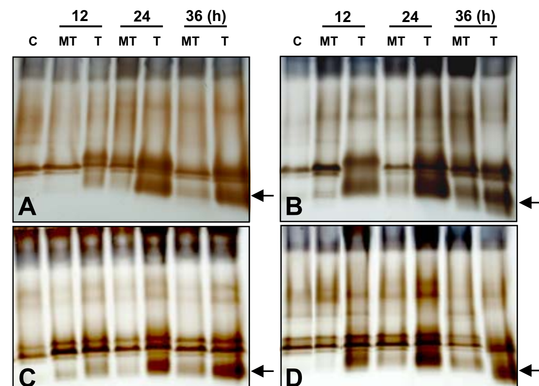


Fig. 2. Native gel analysis for POD activity in four cultivars of sweetpotato (*I. batatas*) after infection with *P. chrysanthemi*. A, cv. Zami; B, cv. Yulmi; C, cv. Shinwhangmi; D, cv. White Star. C, MT and T represent non-treated control, MT and pathogen treatment, respectively. Fifty micrograms of protein was loaded on each gel and electrophoresis and electrophoresis was conducted for 4 h. Arrowheads indicate a novel isoenzyme induced by pathogen infection.

might contribute to chemical as well as physical defenses [18]. The increased POD activity in sweetpotato plants upon infection of *P. chrysanthemi* also supports the involvement of plant PODs in defense.

### 2.2. Differential expression of 10 POD genes in four cultivars after bacterial infection

Response of 10 POD genes isolated from cell cultures of sweetpotato were investigated in four cultivars of sweetpotato after infection of a pathogen by northern analysis. Total RNA was extracted from leaf discs at 20 HAI and hybridized to the 3′-untranslated regions of each cDNA as a specific probe. The 10 POD genes showed diverse expression patterns upon infection with *P. chrysanthemi* in all cultivars (Fig. 3). Several POD genes such as *swpa2, swpa3, swpa4, swpa5*, and *swpb1* were induced upon bacterial infection (T), but other genes were not. Particularly, *swpa4* gene was markedly expressed in response to bacterial infection regardless of cultivar, suggesting that this gene has a stress-inducible promoter. This gene expression was also slightly increased in MT by wounding [26]. The induced gene expression in MT was well matched with the induction by wounding in the previous studies [12,15,26]. Strong induction by wounding was shown in *swpa4*, whereas no induction showed in *swpa1* and *swpa3*. These results indicated that some POD genes contribute to a defense mechanism against pathogen attack. From the results of native gel (Fig. 2) and northern analysis

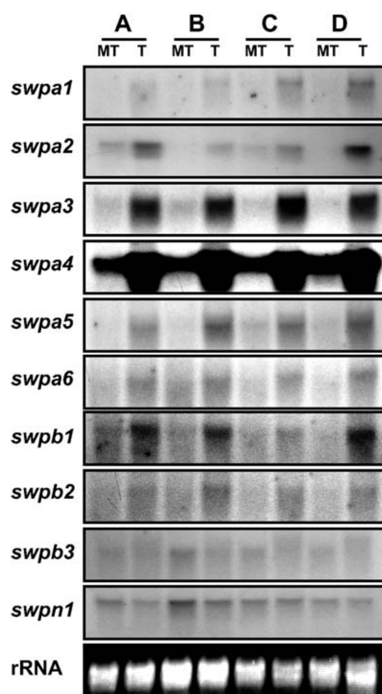


Fig. 3. Expression of 10 sweetpotato POD genes in four cultivars of sweetpotato (*I. batatas*) after infection with *P. chrysanthemi*. A, cv. Zami; B, cv. Yulmi; C, cv. Shinwhangmi; D, cv. White Star. Total RNA was extracted 20 h after pathogen inoculation. MT and T represent mock-treatment and pathogen treatment, respectively. Ethidium bromide-stained RNA served as a loading control.

(Fig. 3), we can speculate that *swpa4* POD might be equivalent to POD isoenzyme highly induced upon pathogen infection.

A particularly interesting finding of this study was the detection of a massive induction of mRNA of *swpa4* in response to pathogen infection. The *swpa4* gene also strongly responded to various abiotic stresses including wounding stress [26]. This result indicates that the *swpa4* gene is involved in multiple stress tolerance, both biotic stress and abiotic stress.

### 2.3. Expression pattern of swpa4 in two cultivars during bacterial infection

The expression patterns of the *swpa4* gene in two sweetpotato cultivars (Zami and White Star) were examined in detail during pathogen infection by northern analysis (Fig. 4). Total RNA from leaf discs was extracted at different times (8, 20, 32 and 44 h) after inoculation with bacteria and hybridized to the 3′-untranslated region of the cDNA as a specific probe. This gene was highly expressed in both cultivars, even though expression patterns were slightly different between two cultivars. In cv. Zami, the transcript was strongly detected from 8 to 32 HAI, and then slightly decreased at 44 HAI. The expression of the *swpa4* gene in White Star showed a weak level at 8 HAI and reached a maximum level at 20 HAI, and then decreased with time. These results very well reflect the difference of POD activity in two cultivars (Zami and White Star) upon pathogen infection (Fig. 1). POD activity in cv. Zami continuously increased to 36 HAI, whereas that in cv. White Star reached a maximum level at 24 HAI, and then decreased.

In this study, the expression of sweetpotato POD genes showed diverse patterns upon pathogen infection, suggesting that some specific POD isoenzymes are involved in defense in relation to pathogenesis of *P. chrysanthemi* in sweetpotato plants. Of the 10 POD genes investigated, the *swpa4* gene was markedly expressed in response to pathogen infection, indicating that this gene has a strong biotic stress-inducible promoter. This promoter will be useful for study of plant–pathogen interaction as well as for the development of trans-

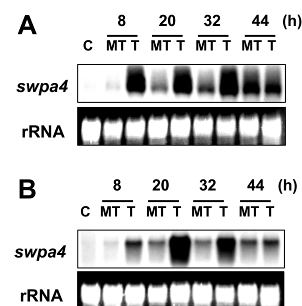


Fig. 4. Expression of *swpa4* gene in two cultivars of sweetpotato (*I. batatas*) after infection with *P. chrysanthemi*. A, cv. Zami; B, cv. White Star. Total RNA was extracted 8, 20, 32 and 44 h after pathogen inoculation. C, MT and T represent non-treated control, MT and pathogen treatment, respectively. Ethidium bromide-stained RNA served as a loading control.

genic plants with enhanced tolerance to multiple stresses including pathogen. *SWPA4* genomic clone encoding *swpa4* POD was cloned and its promoter analysis is under study in terms of external stresses including biotic stress.

## 3. Conclusion

To understand the function of each POD (EC 1.11.1.7) in terms of biotic stress, changes in POD specific activity and expression of 10 POD genes were investigated in four cultivars of sweetpotato (*I. batatas*) after infection with *P. chrysanthemi*. Among 10 POD genes, several POD genes such as *swpa2, swpa3, swpa4, swpa5, swpb1* were induced upon bacterial infection, but other genes were not. Particularly, *swpa4* gene was markedly expressed in response to bacterial infection in four different cultivars, suggesting that this gene has a stress-inducible promoter. The identification of exact POD isoenzyme equivalent to *swpa4* remains to be studied. These results indicate that some specific POD isoenzymes are involved in defense in relation to pathogenesis of *P. chrysanthemi* in sweetpotato plants. Further investigations to understand the function of each POD in terms of various biotic stresses is under study. We are trying to isolate the genomic clone encoding *swpa4* POD to analyze its promoter in relation to biotic stress.

## 4. Methods

### 4.1. Plant material and bacteria inoculation

Four cultivars of sweetpotato (*I. batatas*, Yulmi, Zami, White Star and Shinwhangmi), were obtained from Mokpo Experiment Station, Rural Development Administration, Muan, South Korea. Sweetpotato plants were grown in the greenhouse for 1 month and the third leaves from the top were used for pathogen infection. The bacterial strain used in this study is *P. chrysanthemi* (*Erwinia chrysanthemi*, KCTC 2569), causing bacterial stem and root rot of sweetpotato. *P. chrysanthemi* was grown on nutrient agar medium at 30 °C. For infection with *P. chrysanthemi*, nine leaf discs (18 mm in diameter) placed in a Petri-dish (87 × 15 mm) were inoculated with *P. chrysanthemi* ($1.3 \times 10^4$ cells per ml). Bacterial cell numbers were estimated by measuring the optical density at 600 nm. For MT, leaf discs were treated with distilled water.

### 4.2. POD activity and native gel analysis

For analysis of POD activity and native gel assay, leaf discs of four cultivars were harvested 0, 12, 16, 20, 24 and 36 h post-inoculation. The leaf discs (1 g fr. wt) were homogenized on ice with a mortar in 0.05 M potassium phosphate buffer (pH 7.0, 1/2.5, w/v). The homogenate was centrifuged at 12 000 × *g* for 15 min at 4 °C. The supernatant was used immediately for enzyme assays. Protein concentrations were determined according to the Bradford [2] method using Bio-Rad protein assay reagents and serum albumin as a standard. POD activities (EC 1.11.1.7) were assayed according to the method described by Kwak et al. [20] using pyrogallol as substrate. The standard assay reaction mixture contained, in a total volume of 3 ml: 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 $H_2O_2$ (0.16 ml) and $H_2O$ (2.1 ml). The reaction was initiated by the addition of $H_2O_2$ and the increase in $A_{420}$ nm was recorded in 20 s. One unit of POD activity is defined as that forming 1 mg of purpurogallin from pyrogallol in 20 s at pH 6 at 20 °C. POD gel assays were conducted according to Huh et al. [13] with modifications. The native-polyacrylamide gel electrophoresis (PAGE) was conducted for 4 h at 30 mA using a 12.5% gel. After electrophoresis, POD was stained with 1% benzidine and 1.5% $H_2O_2$.

### 4.3. Northern analysis

Leaf discs were harvested for RNA isolation at 0, 8, 20, 32 and 44 h after inoculation with bacteria or distilled water. Total RNA was extracted from plant materials by the LiCl method [25], denatured by heating at 70 °C for 15 min in a denaturation buffer containing formamide–$H_2O$ (1/1, v/v) and 2.2 M formaldehyde, and blotted onto a Zeta-probe GT membrane (Bio-Rad). The blots were probed with $^{32}$P-labeled gene-specific DNA fragments from the 3′-untranslated sequence specific to their own cDNAs. Hybridization was carried out in 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM EDTA at 65 °C. Gene-specific primers for PCRs were designed from the 3′-untranslated regions or regions near the translation stop codon of each gene [26].

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