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# Molecular characterization of the sweet potato peroxidase *SWPA4* promoter which responds to abiotic stresses and pathogen infection

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Previously, the *swpa4* peroxidase gene has been shown to be inducible by a variety of abiotic stresses and pathogenic infections in sweet potato (*Ipomoea batatas*). To elucidate its regulatory mechanism at the transcriptional level under various stress conditions, we isolated and characterized the promoter region (2374 bp) of *swpa4* (referred to as *SWPA4*). We performed a transient expression assay in tobacco protoplasts with deletions from the 5′-end of *SWPA4* promoter fused to the β-glucuronidase (GUS) reporter gene. The −1408 and −374 bp deletions relative to the transcription start site (+1) showed 8 and 4.5 times higher GUS expression than the cauliflower mosaic virus *35S* promoter, respectively. In addition, transgenic tobacco plants expressing GUS under the control of −2374, −1408 or −374 bp region of *SWPA4* promoter were generated and studied in various tissues under abiotic stresses and pathogen infection. Gel mobility shift assays revealed that nuclear proteins from sweet potato cultured cells specifically interacted with 60-bp fragment (−178/−118) in −374 bp promoter region. In silico analysis indicated that four kinds of *cis*-acting regulatory sequences, reactive oxygen species-related element activator protein 1 (AP1), CCAAT/enhancer-binding protein alpha element, ethylene-responsive element (ERE) and heat-shock element, are present in the −60 bp region (−178/−118), suggesting that the −60 bp region might be associated with stress inducibility of the *SWPA4* promoter.

## Introduction

Plant peroxidases (PRXs, EC 1.11.1.7) catalyze the oxidoreduction of various substrates, such as phenolics, lignin precursors and secondary metabolites using hydrogen peroxide ($H_2O_2$) (Hiraga et al. 2001, Passardi et al. 2005). Secretory plant PRXs are member of a large multigenic family in plant species. Diversity of the reactions catalyzed by PRXs describes the implication of these proteins in a broad range of physiological processes, such as auxin metabolism, lignin and suberin formation, cross-linking of cell wall proteins and defense responses against environmental stresses (Hiraga et al. 2001, Passardi et al. 2005). The complete analysis of the *Arabidopsis* and rice genomic sequence has revealed the

*Abbreviations* – BY-2, Bright Yellow 2; C/EBPα, CCAAT/enhancer-binding protein alpha; CaMV, cauliflower mosaic virus; CURE, copper-responsive element; ERE, ethylene-responsive element; GSPs, gene-specific primers; GUS, β-glucuronidase; HSE, heat-shock element; LUC, luciferase; MV, methyl viologen; PCR, polymerase chain reaction; PRX, peroxidase.

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presence of 73 PRX genes in *Arabidopsis thaliana* and 138 PRX genes in rice (Passardi et al. 2004, Tognolli et al. 2002). However, the presence of multiple isoforms and the complexity of physiological processes make it difficult to understand the specific function of PRX enzymes in planta and their specific roles in plant development, growth and adaptation to environment. Information on the timing, level and tissue specificity of each PRX gene expression is important to understand the specific role of each PRX enzyme. For example, the wound-inducible tobacco *tpoxN1* PRX gene was expressed not only in the vascular system of stems and petioles but also in the epidermal system, indicating the vascular system specificity of *tpoxN1* PRX gene (Sasaki et al. 2002). The expression profiles of 10 rice PRX genes were analyzed in response to blast fungus infection (Sasaki et al. 2004). In our previous studies, 10 PRX cDNAs were isolated from cell cultures of sweet potato, and their expression levels were characterized to understand the physiological functions of each PRX in response to various stresses (Huh et al. 1997, Jang et al. 2004, Kim et al. 1999, 2007, Park et al. 2003). Among these PRX genes, the transcript expression of *swpa4* PRX gene was strongly induced by various abiotic stresses and pathogen infection compared with other PRX genes (Jang et al. 2004, Kim et al. 2007, Park et al. 2003).

A powerful expression system with an appropriate promoter is an important requisite for efficient expression of foreign genes in plant cells. In most experiments, the transgenes are driven under the control of a strong promoter, such as the *35S* promoter of the cauliflower mosaic virus (CaMV). However, a more conditional gene expression system is needed to extract greater benefits from transgenic technology (Aoyama and Chua 1997, Kasuga et al. 1999). Furthermore, development of stress-inducible promoters that control precisely the expression of target defense genes under particular stress conditions is very important for developing transgenic plants with an enhanced tolerance to multiple stresses.

We have isolated previously a strong oxidative stress-inducible PRX (*SWPA2*) promoter from cultured cells of sweet potato, and its function was characterized in transgenic tobacco plants and cultured cells under environmental stress conditions, such as $H_2O_2$, wounding and UV treatment (Kim et al. 2003). Accumulating evidence suggests that stress-inducible *SWPA2* promoter is applicable to other plant systems for the development of stress-tolerant transgenic plants. Use of *SWPA2* promoter to conditionally induce the expression of antioxidant genes in several plant systems made it possible to develop transgenic plants with an increased tolerance to multiple stresses (Ahmad et al. 2008, Lee et al. 2007, Lim et al. 2007, Tang et al. 2006, 2007).

We have reported previously that transgenic tobacco plants overexpressing the *swpa4* gene exhibited increased tolerance to various abiotic and biotic stresses (Kim et al. 2008). To get an insight into the regulatory mechanisms underpinning *swpa4* gene under various stress conditions, we have isolated the 5′-regulatory region upstream of transcription start site of *swpa4* gene. In this study, we describe a new multiple stress-inducible PRX promoter from sweet potato and its subsequent characterization using an in vitro transient assay and stable transgenic tobacco plants. Our results suggest the potential utility of the *SWPA4* promoter to develop transgenic plants with an increased tolerance to environmental stresses.

## Materials and methods

### Chromosome walking and genomic polymerase chain reaction

To isolate the promoter region for the *swpa4* PRX gene, genomic walking was performed with the Universal GenomeWalker Kit (Clontech, Tokyo, Japan) according to the manufacturer's guidelines. Briefly, genomic DNA (2.5 μg) of sweet potato (*Ipomoea batatas* L. Lam. cv. White star) was digested with *Eco*RV, *Dra*I, *Pvu*II and *Stu*I. DNA fragments were ligated with a GenomeWalker adaptor, which had one blunt end and one end with a 5′-overhang. The ligation mixture of the adaptor and the genomic DNA fragments were used as a template for polymerase chain reaction (PCR). Primary and secondary (nested) PCR was performed using Advantage 2 Polymerase Mix (Clontech) with adaptor primers and *swpa4* gene-specific primers (GSPs) according to the manufacturer's guide. The PCR products were fractioned to agarose gel, and the amplified DNA (over 2 kb) were purified and cloned in pGEM-T Easy vector (Promega, Madison, WI). The complete sequence was determined by sequencing. Two *swpa4* GSPs are as follows: GSP1 (5′-CTGAGCCGAGTGACAAAGGAAGCCAT-3′) and GSP2 (5′-GTAATACGACTCACTATAGGGC-3′). To obtain the genomic clone corresponding to the *swpa4* cDNA, we performed genomic PCR using sweet potato (cv. White star) genomic DNA with a pair of primer synthesized based on the *swpa4* cDNA sequence (swpa4-For, 5′-ATGGCTTCCTTTGTCACTCGGCTCAG-3′; swpa4-Rev, 5′-CATGGATGCAACTGTGTTGGC-3′).

### Plasmid construction

For the analysis in stable transgenic plants and the transient expression assay, the *SWPA4* promoter-β-glucuronidase (GUS) reporter-NOS terminator cassette was constructed. A *Hin*dIII/*Xba*I fragment of *SWPA4*

promoter amplified by PCR was cloned into the same site of pBI221 vector for transient assay and was introduced into the pBI101 vector for generation of stable transgenic plants (Clontech). A series of deletion vector constructs of *SWPA4* promoter was generated by PCR amplification. Each primer of fragments, named 51, 118, 178, 247, 307, 374, 759, 1140, 1408, 1875 and 2374, was synthesized based on the nucleotide sequence of the g*SWPA4* genomic clone. The upstream primers were designed to contain the *Hin*dIII restriction enzyme site for cloning. The downstream primer was synthesized to introduce the *Xba*I restriction enzyme site. The purified PCR products were digested with *Hin*dIII/*Xba*I and cloned into the pBI221 and pBI101 binary vectors.

### Transient transformation with tobacco Bright Yellow 2 cells

Suspension-cultured cell line of tobacco Bright Yellow 2 (BY-2) (*Nicotiana tabacum* L. cv. Bright Yellow 2) was used for transient expression by PEG method as described previously by Nagata (1987). Typically, 0.3 ml of protoplast suspension culture ($5 \times 10^6$ ml$^{-1}$) was cotransfected with both 15 μg of deletion vector constructs and 5 μg of CaMV *35S* promoter-luciferase (LUC) control vector. The transfected protoplasts were incubated in W5 solution for 18 h in the dark at room temperature. For normalization of transfection efficiency, CaMV *35S* promoter-LUC control vector was cotransfected in each experiment. LUC assays were performed using the Luciferase Assay System (Promega) according to the manufacturer's instruction.

### Fluorometric and histochemical analysis of GUS activity

Histochemical and fluorometric analysis of GUS activity was essentially performed as described by Jefferson et al. (1987). For the fluorometric assay, GUS activity in crude extracts was assayed using 4-methyl umbelliferyl glucuronide as a substrate. Histochemical staining for GUS activity was performed with X-gluc as a substrate. Protein content of sample extracts was determined according to the method of Bradford (1976) using the Protein Assay Kit (Bio-Rad, Hercules, CA) with BSA as a standard. Data are presented as the mean of GUS activity from at least three independent determinations.

### Preparation of transgenic tobacco plants

For the generation of transgenic tobacco (*N. tabacum* cv. Xanthi) plants, the constructed plasmids of p2,374, p1,408, p374 and pBI121 were introduced into *Agrobacterium tumefaciens* EHA105, and transgenic plants were generated according to the leaf disc method as described by Kim et al. (2003). Transformants were selected on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) medium supplemented with 200 mg l$^{-1}$ kanamycin and 300 mg l$^{-1}$ cefotaxime. Kanamycin-resistant shoots were directly formed on cut edges of leaf discs. Rooting of kanamycin-resistant plantlets was carried out in the selection MS medium without any growth regulators.

### Stress treatments

For stress treatment, the third and fourth fully expanded leaves from the top of 4-week-old tobacco plants grown in a growth chamber were used. For $H_2O_2$ treatment, seven leaf discs (7 mm in diameter) were floated on 50 m*M* $H_2O_2$ in a Petri dish under continuous white light at 25°C for 48 h and then the GUS activity was measured. For NaCl treatment, leaf discs were floated on 200 m*M* NaCl in a Petri dish under continuous white light at 25°C for 24 h. For wounding stress, leaf discs were wounded by pressing with a needle puncher as described previously by Huh et al. (1997), and GUS activity was measured after keeping them in growth chamber for 48 h. For pathogen treatment, *Pseudomonas syringae* pv. *tabaci* ($10^5$ cells ml$^{-1}$ in 10 m*M* $MgCl_2$) was inoculated to the leaves of tobacco by infiltration. Mock-inoculated tissues were infiltrated with 10 m*M* $MgCl_2$ solution. Mock-inoculated and pathogen-inoculated plants were kept in the growth chamber, and the leaves were collected at 48 h later and immediately frozen in liquid nitrogen for GUS activity assay.

### Gel mobility shift assay

The Sigma Cell Lytic Plant Nuclei Isolation/Extraction Kit (Sigma, St Louis, MO) was used according to the manufacturer's instructions to obtain nuclear protein extracts from the suspension-cultured cells and the leaves of sweet potato. Protein content of nucleic extracts was determined according to the method of Bradford (1976) using the Protein Assay Kit (Bio-Rad). DNA probe labeling and the gel mobility shift assay were performed with the Gel Shift Assay System (Promega). DNA probes were labeled at the 5′-end by T4 polynucleotide kinase in the presence of [γ-$^{32}$P] ATP. The $^{32}$P-labeled DNA probes were purified using Bio-Spin column (Bio-Rad) and were quantified with a LSC counter (Beckman, Palo Alto, CA). Nuclear extracts (10 μg) were incubated in a gel shift binding buffer [5% glycerol; 1 m*M* $MgCl_2$; 0.5 m*M* EDTA; 0.5 m*M* DTT; 50 m*M* NaCl; 10 m*M* Tris–HCl, pH 7.5; 0.05 mg ml$^{-1}$ poly(dI-dC)·poly(dI-dC)] for 30 min at room temperature with 40 000 cpm of end-labeled DNA probes. The reaction

mixtures were subjected to electrophoresis on a non-denaturing 5% polyacrylamide gel. The gel was dried and subjected to autoradiography. The excess of unlabeled competitor (5-fold, 10-fold and 100-fold molar excess) of double-stranded DNA was used for competition experiment. *Swpa2* consensus oligo (swpa2-For, 5′-CATTAA-ACAGAAAAGAAAGGAAAAAATGACTCACCACCTAG-3; swpa2-Rev, 5′-CATCTAGGTGGTGAGTCATTTTTTCCT-TTCTTTTCTGTTTAAT-3′), which is the consensus oligonucleotide sequence that swpa2 promoter regions binds as positive control. AP1 consensus oligo (AP1-For, 5′-CGCTTGATGAGTCAGCCGGAA-3′; AP1-Rev, 5′-GCG-AACTACTCAGTCGGCCTT-3′), which is the consensus oligonucleotide sequence that AP1 (c-Jun) transcription factor binds, was used as a non-specific oligonucleotide.

### Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from the suspension-cultured cells and the leaves of sweet potato using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription-polymerase chain reaction (RT-PCR) amplification was conducted using an RT-PCR kit (Promega) in accordance with the manufacturer's instructions. Total RNA (1 μg) was utilized for the generation of first-strand cDNA using Moloney marine leukaemia virus (MMLV) reverse transcriptase. To amplify a 174-bp product from cDNA coding for *swpa4* by PCR, the *swpa4* GSPs were as follows: *swpa4*-For primer (5′-CAGCGAGGTGAATGCCAACA-3′), *swpa4*-Rev primer 5′-TTCAGTAATACAAGTTTTGT-3′). As an internal control of reverse transcription, 18S internal standards (Ambion, Austin, TX) were used.

## Results

### Isolation and transient expression assay of *SWPA4* promoter

As a first step to study the regulatory mechanism controlling expression of *swpa4* gene in response to various stresses, we isolated the genomic clone, named

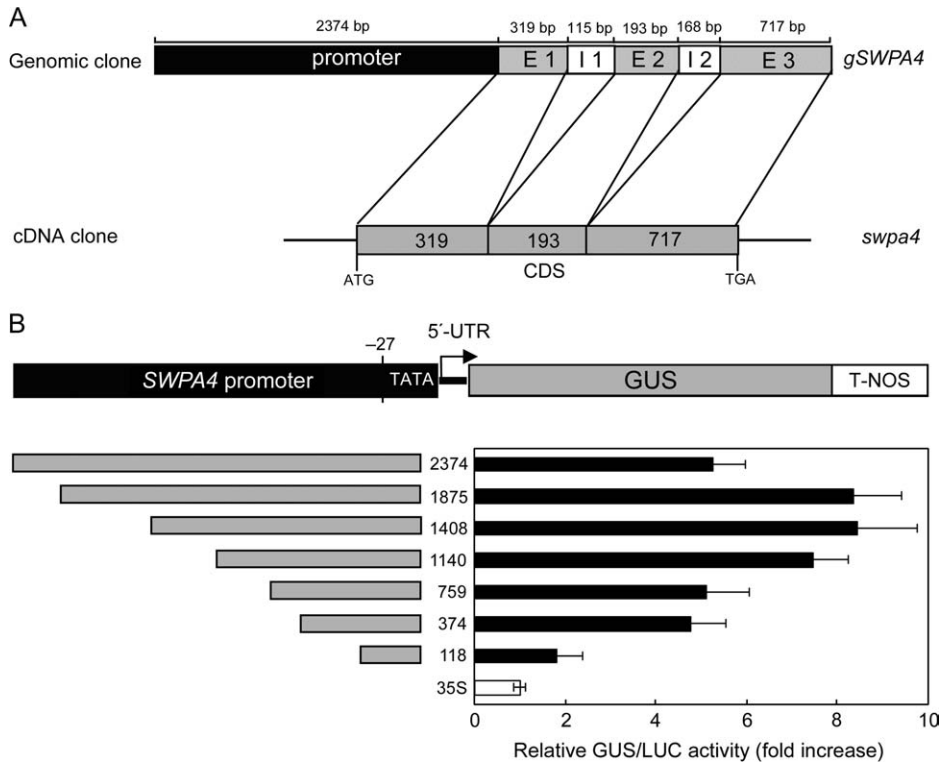


**Fig. 1.** Isolation of *swpa4* peroxidase gene from sweet potato. (A) Schematic representation of the *gSWPA4* genomic clone (accession number: DQ676955) and its corresponding cDNA (*swpa4*). Exons and introns are denoted by E and I, respectively. The numbers above the exons and the introns state their sizes in nucleotides. (B) Functional analysis of *SWPA4* promoter deletion fragments in tobacco BY-2 protoplasts. Deletion constructs of *SWPA4* promoter were fused to the GUS gene in pBI221 vector. The seven deletion constructs containing different *SWPA4* 5′-regulatory regions were analyzed by transient expression. The numbers in the diagram refer to the 5′-end of the *SWPA4* fragments upstream of the transcription start site. Deletion fragments are defined as numbers of base pairs from the transcription start site. GUS activity was expressed relative to that supported by CaMV *35S* promoter. LUC activity was used as an internal standard for normalization. Data are mean ± SE of six replicates. CDS, coding sequence.

g*SWPA4*, corresponding to *swpa4* cDNA by chromosome DNA walking approach with a long-distance PCR. For genomic walking of the promoter region, gene-specific primers were designed, such as GSP1 and GSP2 (Supporting information Fig. S1). We were able to amplify several fragments for each region with different lengths from four individual libraries. Through subsequent restriction and sequence analysis, we chose the largest fragments and cloned them into pGEM-T easy vector and then the complete sequence was determined. The *gSWPA4* genomic clone consists of 2374 bp of 5′-regulatory region upstream of transcription start site and 1512 bp of the coding region (Fig. 1A). Comparison of the genomic and the cDNA sequence revealed that the coding region of *swpa4* consist of three exons of 319, 193 and 717 bp separated by two introns of 115 and 168 bp. The positions of the introns were localized by comparing the *gSWPA4* genomic sequence with the *swpa4* cDNA. The nucleotide sequence of the coding region of the *gSWPA4* genomic clone exactly matched that of the *swpa4* cDNA clone. The coding region of the first reported sweet potato PRX gene, *swpa2*, also was shown to contain three exons and two introns (Kim et al. 2003). The deduced amino acid sequences of *swpa2* and *swpa4* showed 71% sequence homology, whereas a very low homology (around 35%) between both promoter regions was observed. Among the plant PRX genes in the databases, *swpa4* is most closely related to the tomato TMP1 and TMP2, showing 53 and 55% sequence identity at the amino acid level. The *TMP* genes were induced by ABA treatment in tomato callus. In addition, ABA-stimulated suberization was shown to lead to the induction of the *TMP* gene expression (Roberts and Kolattukudy 1989). The transcribed region of the *swpa4* gene was deduced by the direct sequencing of 5′-RACE product (data not shown). Sequence analysis of the RACE product indicated that the 5′-end of the *swpa4* transcript was located 59 bp upstream of the translation initiation site of the *swpa4* gene (Fig. 1A).

To investigate the promoter activity of the *swpa4* gene, seven different sizes of *SWPA4* promoter were fused to the GUS reporter gene (Fig. 1B). The CaMV *35S* promoter and deletions of the *SWPA4* promoter were analyzed by transient expression assay employing protoplast cultures of tobacco BY-2 cells. The activities of *SWPA4* promoter deletions were examined by measuring the activity of the GUS reporter enzyme. A *LUC* reporter plasmid was cointroduced as an internal control, and the GUS activity of each sample was normalized using LUC activity as standard. The −1875 and −1408 bp of deletion fragments exhibited the highest levels of GUS activity, which was about 8.5 times higher relative to the CaMV *35S* promoter. The level of GUS expression reached a maximum at −1408 bp of deletion fragment and then decreased at −759 bp of deletion fragment. The −374 bp of deletion showed about five times higher level of GUS activity than the *35S* promoter. These results suggest that certain enhancer sequences for expression in BY-2 cell culture may be located between the promoter regions −1875 and −759 bp upstream of the transcription start site, whereas negative *cis*-elements may exist between −2374 and −1875 bp. The −374 bp region upstream of the transcription start site is likely responsible for the expression of *SWPA4* promoter in suspension-cultured cells.

## Analysis of *SWPA4* promoter in stable transgenic tobacco plants

To examine the *SWPA4* promoter activity in transgenic tobacco under various stress conditions, stable transgenic tobacco plants with the *SWPA4* promoters of −2374, −1408 and −374 bp were generated by agrobacterium-mediated transformation. $T_1$ transgenic tobacco lines were confirmed by PCR and Southern blot analysis for the presence of the respective *SWPA4*::*GUS* and CaMV

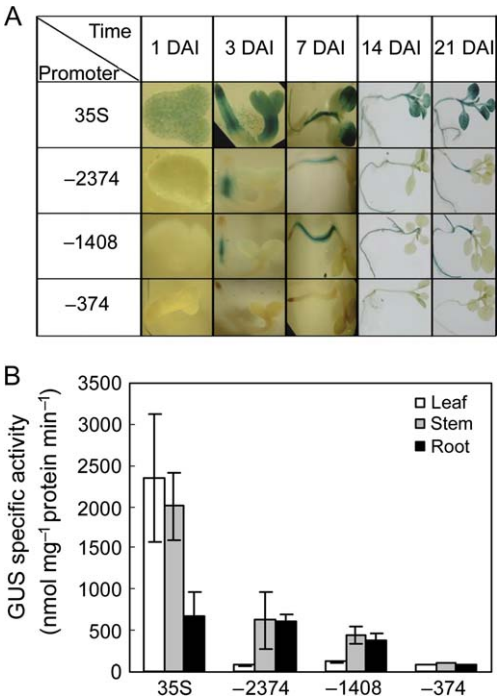


**Fig. 2.** GUS expression analysis of three deletion constructs of *SWPA4* promoter::*GUS* in seedlings of transgenic tobacco plants. (A) GUS staining from 1 to 21 days after imbibition (DAI) in seedlings of transgenic tobacco plants. (B) GUS activity assay in 21-day-old seedling tissues after imbibition. The three deletion constructs of *SWPA4* promoter (−2374, −1408 and −374 regulatory regions) fused to GUS were analyzed for GUS expression. The *35S* promoter::*GUS* construct was used as a positive control. Data are mean ± SE of three replicates.

*35S*::*GUS* cassettes (data not shown). The GUS activity in transgenic lines of CaMV *35S* promoter was well observed in all the tissues of seedlings during 21 day after imbibition. Both −2374 and −1408 deletion promoters induced the GUS expression in stem and root tissues of young seedlings, but the GUS activity was not detectable in seedlings of −374 bp promoter (Fig. 2A). Fig. 2B shows the GUS activities of CaMV *35S* and three *SWPA4* deletion promoter plants in tissues of leaf, stem and root. These results indicate that positive regulatory sequences for tissue-specific expression are located between −1408 and −374 bp region of *SWPA4* promoter.

Our previous results showed that *swpa4* strongly responded under various abiotic stresses and pathogen infection in sweet potato (Jang et al. 2004, Kim et al. 2007, Park et al. 2003). In silico analysis revealed that the 2374 bp region of *SWPA4* promoter contains several stress-related *cis*-elements, such as reactive oxygen species-related element (AP1), CCAAT/enhancer-binding protein alpha (C/EBPα) element, gibberellin-responsive element (GARE), CAACTC regulatory elements (CARE), copper-responsive element (CURE), ethylene-responsive element (ERE), GCN4 protein binding element (GCN4), heat-shock element (HSE), low temperature-responsive element, dehydration-responsive element (MYB recognition site), cold-responsive element (MYC recognition site) and pathogen-responsive element (W-box) (Table 1). Therefore, we further determined GUS activity in the leaves of $T_1$

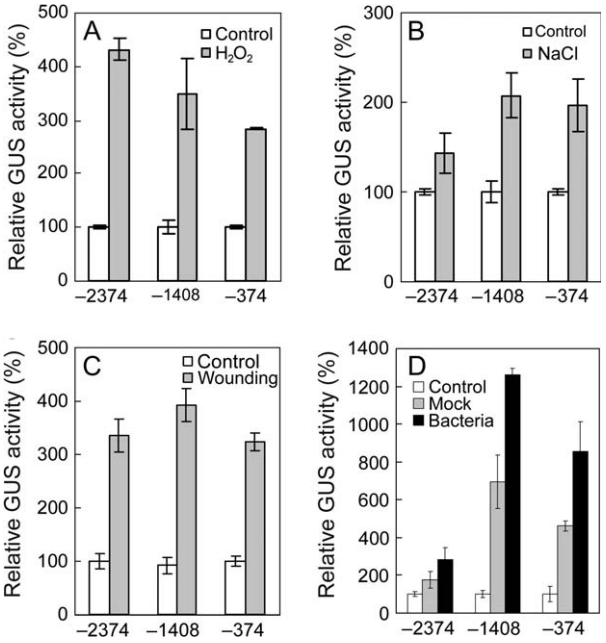


**Fig. 3.** GUS activity assay of the deletion fragments of *SWPA4* promoter in response to various stress conditions. Transgenic tobacco plants expressing the three deletion fragments of *SWPA4* promoter fused to GUS were generated. Two-month-old plants were assayed for the GUS activity following various treatments. (A) $H_2O_2$ treatment (50 m*M*, 24 h), (B) NaCl treatment (200 m*M*, 24 h), (C) mechanical wounding (48 h), (D) bacterial pathogen (*Pseudomonas syringae* pv. *tabaci*, $10^5$ cells $ml^{-1}$, 48 h). Data are mean ± SE of three independent replicates.

**Table 1.** Putative *cis*-elements found in −2374 bp region of the *SWPA4* promoter. The data have been obtained with PLACE and TESS. Indicated positions are relative to the transcription start site. CBF, C-repeat binding factor. WRKY, WRKY domain protein. SA, Salicylic acid.

| Motif | Sequence | Function | Position |
|---|---|---|---|
| AP1 homology | RSTGACTMANN | Reactive oxygen species-related *cis*-element | −967, −468, −358, −213, −169 |
| CAAT-box | CAAT | CAAT promoter consensus sequence | −77 |
| CARE | CAACTC | Gibberellin inducible | −1718, −455, −424 |
| CURE | GTAC | Copper response | −1485, −835, −769, −551, −316, −179 |
| C/EBPα | CCAAT | C/EBPα response | −1275, −1139, −177, −149 |
| DPBF core | ACACNNG | ABA response | −1802 |
| ERE | AWTTCAAA | Ethylene response | −126 |
| GARE | TAACGTA | Gibberellin response | −317 |
| GCN4 | TGAGTG | Oxidative stress response | −468, −370 |
| HSE | AGAAC | Heat-shock response | −2139, −1582, −588, −119, −52, −35 |
| LTRE | CCGAAA | Low temperature response | −1560 |
| MYB1 consensus | WAACCA | Myb recognition, dehydration inducible | −1644, −1354 |
| MYB2 consensus | YAACKG | Myb recognition, dehydration inducible | −1597, −675 |
| MYB core | CNGTTR | Myb recognition, dehydration inducible, flavonoid biosynthesis | −311 |
| MYC consensus | CANNTG | CBF inducer, cold inducible | −2063, −1960, −1802, −1678, −1518, −942, −935, −387 |
| TATA-box | TATTTAA | TATA-box | −27 |
| W-box | TGAC | WRKY recognition, SA-inducible, wounding inducible, pathogen inducible | −1930, −1159, 969, −940, −367, −336, −213 |

transgenic tobacco plants under various stress conditions, such as $H_2O_2$, NaCl, wounding and pathogen (Fig. 3). $H_2O_2$ treatment to all of *SWPA4* promoter transgenic lines resulted in three-fold or four-fold increase in GUS activity compared with the untreated control (Fig. 3A). The GUS activities increased about two times at 24 h after NaCl treatment and enhanced three or four times at 48 h after wounding treatment (Fig. 3B, C). Inoculation of the bacterial pathogen, *P. syringae* pv. *tabaci*, induced about two-fold increase in the GUS activities at 48 h compared with mock treatment (Fig. 3D). Mock inoculation itself also caused an increase in the GUS activities because *swpa4* gene is responsive to wounding as shown in Fig. 3C.

Unlike −2374 and −1408 promoter regions, the −374 bp region did not exhibit tissue-specific activity in root and stem (Fig. 2). However, the −374 region showed similar GUS activity to the −2374 bp promoter region in suspension-cultured cells (data not shown) and under stress conditions (Fig. 3). This indicates that the −374 bp regulatory region is sufficient to confer the inducibility of *swpa4* gene in response to abiotic stresses and pathogen infection.

## Functional analysis of 60 bp region in *SWPA4* promoter

To investigate the minimal regulatory sequence required for a high expression of *SWPA4* promoter in suspension-cultured cells, we further carried out transient assay with 5′-deletion fragments of the −374 bp region (Fig. 4A).

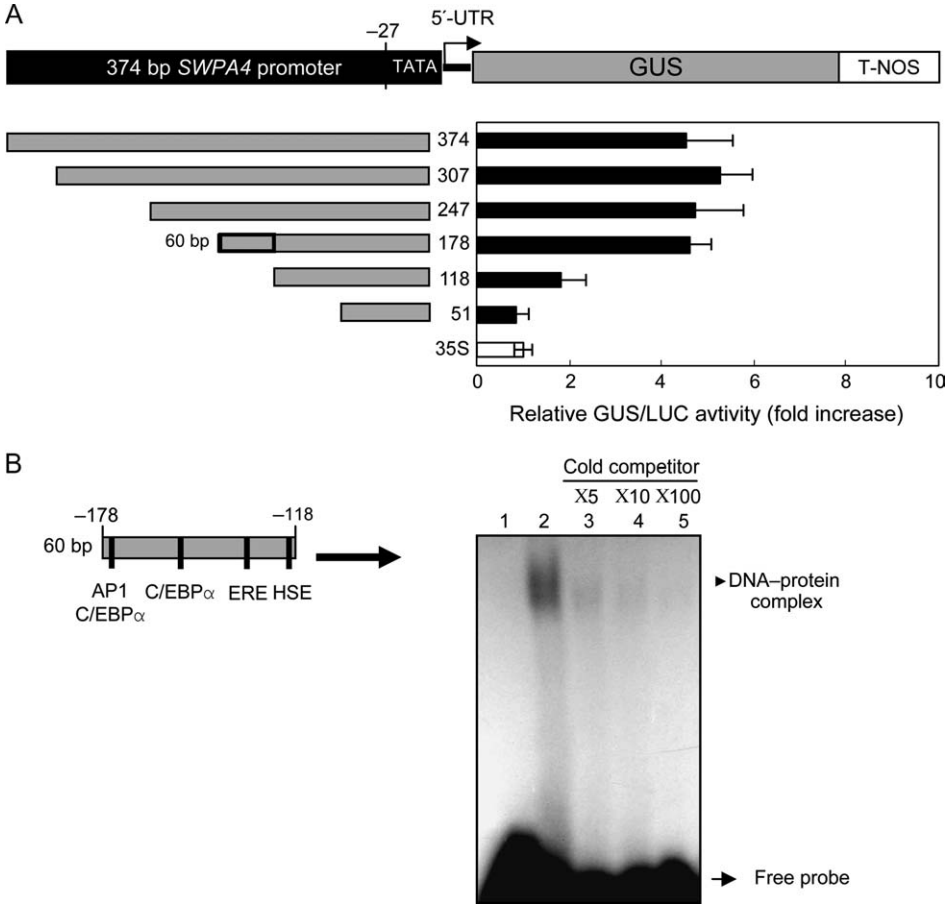


**Fig. 4.** GUS expression analysis of −374 bp deletion fragments of *SWPA4* promoter and the enhanced DNA-binding activity of −60-bp fragment. (A) Transient expression assay of −374 bp deletion constructs of *SWPA4* promoter in tobacco BY-2 protoplasts. The numbers in the diagram refer to the 5′-end of the deletion fragments of *SWPA4* promoter upstream of the transcription start site. GUS activity was expressed relative to that supported by CaMV *35S* promoter, and LUC activity was used an internal standard. Data are mean ± SE of six replicates. (B) The induced DNA-binding activity of the −60 bp (−178 to −118 bp) region of the *SWPA4* promoter is specific in sweet potato cultured cells. The stress-related putative *cis*-elements are indicated by lines in gray box: AP1 (reactive oxygen species-related element), C/EBPα, HSE and ERE (ethylene-responsive element). Gel mobility shift assay was carried out with the 60 bp DNA fragment as a probe. The probe was incubated in the absence (lane 1) or presence (lanes 2–5) of the nuclear extracts. Cold competitors were also added as follows: lane 3, 5-fold amount of the 60 bp DNA fragment; lane 4, 10-fold amount of the 60 bp DNA fragment; lane 5, 100-fold amount of the 60 bp DNA fragment. The arrowhead indicates the DNA–protein complex, and the arrow shows the free probe.

We found that the removal of the region between −178 and −118 bp greatly reduced the GUS activity of −118 bp region of *SWPA4* promoter. In silico analysis indicated that five putative *cis*-acting regulatory sequences, such as AP1 (−169), C/EBPα (−149 and −177), HSE (−119) and ERE (−126) are present in the 60 bp region of *SWPA4* promoter. Internal deletion of this region in −374 bp promoter resulted in a decrease in GUS activity (data not shown). These results suggest that the 60 bp region is essential for stress inducibility of *SWPA4* promoter in suspension cells. Cell suspension culture is known to be exposed to higher oxidative stress conditions than whole plants as shown through an evaluation of antioxidant activity (Kwak et al. 1995).

Thus, we further examined whether the 60 bp region is capable of binding nuclear protein factors in suspension-cultured cells of sweet potato (Fig. 4B). Our gel mobility shift assay showed that the 60-bp fragment of *SWPA4* promoter bound to the nuclear extract prepared from sweet potato cultured cells. To verify whether the binding activity is specific, we performed competition experiment using excess amounts of unlabeled DNAs. As shown in Fig. 4B, the binding activity was dramatically reduced in the presence of the unlabeled cold competitor and completely disappeared when the amount of the cold competitor was increased by 100-fold.

We also checked whether the 60 bp region is capable of binding protein factors in leaves of sweet potato under stress conditions (Fig. 5). At first, we performed the RT-PCR analysis to investigate the inducibility of *swpa4* gene in both suspension-cultured cells and methyl viologen (MV)-treated leaves of sweet potato (Fig. 5A). Transcript levels of *swpa4* increased in both suspension cells and MV-treated leaves compared with the leaves of normal conditions. Our gel mobility shift assay showed that the 60-bp fragment of *SWPA4* promoter resulted in higher binding capacity in the suspension cells and the MV-treated leaves compared with the untreated leaves (Fig. 5B). Because enhanced binding activity of *SWPA2* promoter to nuclear extracts from the suspension-cultured cells of sweet potato was detected, we used *SWPA2* promoter region (−24 bp) as a positive control for gel mobility shift assay (Kim 2000). AP1 of HeLa cells was used as a negative control. This result demonstrates that the nuclear factors from sweet potato specifically bind to the 60-bp fragment of *SWPA4* promoter, which contains the putative AP1, C/EBPα, ERE and HSE.

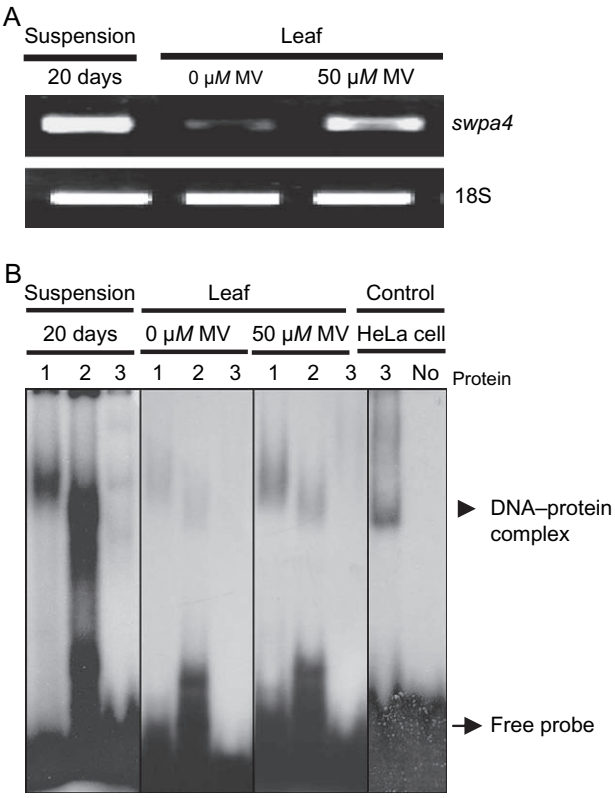


**Fig. 5.** Enhanced DNA-binding activity of −60-bp fragment in suspension-cultured cells and MV-treated leaves of sweet potato. (A) Expression analysis of *swpa4* by RT-PCR in suspension-cultured cells and MV-treated sweet potato leaves. 18S RNA was used as a control for equal loading. (B) Gel mobility shift assay of −60-bp fragment in suspension-cultured cells and MV-treated leaves of sweet potato. AP1 probe was used as a positive control to detect AP1-binding activity in HeLa nuclear extracts. 1, −60 bp region probe of *SWPA4* promoter as a probe; 2, −24 bp region probe of *SWPA2* promoter as a positive control; 3, AP1 consensus probe as a negative control (lane 3). The arrowhead indicates the DNA–protein complex, and the arrow shows the free probe.

## Discussion

We have shown previously that the *swpa4* gene was inducible to oxidative stress in sweet potato (Jang et al. 2004, Kim et al. 2007, Park et al. 2003). In this study, as a step to understand regulatory mechanisms controlling the *swpa4* gene expression, we isolated a promoter region of the *swpa4* gene. Our results demonstrate that the 60 bp promoter region is likely responsible for the inducibility of the *swpa4* gene in response to oxidative stress in the cultured cells of sweet potato.

We noted that *SWPA4* promoter exhibited higher GUS activity than *35S* promoter in suspension-cultured cells (Figs 1 and 4), whereas it showed lower GUS activity than *35S* promoter in plants under normal conditions (Fig. 2). In the previous study, we reported that most *POD* genes including *swpa4* exhibited higher expression levels in suspension-cultured cells than plant tissues (Huh et al. 1997, Kim et al. 1999, Park et al. 2003). This could be explained from the observation that suspension-cultured cells are exposed to higher oxidative stress conditions

than whole plants as judged by evaluation of antioxidant activity (Kwak et al. 1995). The *SWPA2* promoter also showed higher GUS activity in suspension-cultured cells relative to normal plant tissues (Kim et al. 2003).

In our previous report, overexpression of *swpa4* gene induced an increased generation of $H_2O_2$ in transgenic tobacco plants (Kim et al. 2008). Moreover, increased expression of various apoplastic acidic pathogenesis-related (*PR*) genes followed enhanced $H_2O_2$ production. The role of $H_2O_2$ as a key signal in abiotic and biotic stress defenses, such as acclimation to chilling and high light, wounding response and pathogen defense response, has been well documented (Kim et al. 2008, Vranova et al. 2002). Our previous RT-PCR analysis showed that expression of *swpa4* gene was induced by $H_2O_2$ or MV-mediated oxidative stress treatments (Park et al. 2003). In addition, we observed that GUS activity of −374 bp region of *SWPA4* promoter in transgenic tobacco was induced by $H_2O_2$ treatment (Fig. 3). These results suggest that the inducibility of *swpa4* gene in response to $H_2O_2$ might be regulated by activation of $H_2O_2$-stimulated *cis*-elements within −374 bp region of *SWPA4* promoter. By in silico analysis, we found that the −374 bp region of *SWPA4* promoter contains several stress-related *cis*-elements, such as AP1, C/EBPα, CURE, ERE, GARE, GCN4, HSE and W-box (Table 1). Moreover, we observed that 60-bp fragment within −374 bp region was responsible for DNA-binding activity. Based on database analysis, we noted the presence of AP1, C/EBPα, ERE and HSE in the 60-bp fragment, indicating that the these elements might be an important DNA-binding site for transcription factor(s) involved in activation of *swpa4* gene in response to oxidative stress in suspension-cultured cells. In addition, we also do not exclude the possibility that any unknown *cis*-elements might be involved in the oxidative stress response of the *SWPA4* promoter.

Recently, we have developed transgenic sweet potato, potato and tall fescue plants expressing antioxidant genes under the control of *SWPA2* promoter (Ahmad et al. 2008, Lee et al. 2007, Lim et al. 2007, Tang et al. 2006, 2007). These transgenic plants showed enhanced tolerance to multiple stresses, in particular, abiotic stress. Because the *SWPA4* promoter was markedly inducible to pathogen infection as well as under oxidative stresses, it will provide another useful material for development of environment stress-tolerant transgenic plants. Furthermore, further study for isolation of exact *cis*-elements and transcription factors involved in regulating the expression of *swpa4* gene under multiple stress conditions would be useful for the understanding of stress signal transduction pathway in plants.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Nucleotide and deduced amino acid sequences of the *gSWPA4* genomic clone. Arrows indicate the promoter region GSPs used for chromosome walking with a long-distance PCR. Arrowheads show the transcription start site and the 5′-end sequence of *swpa4* cDNA.

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