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ORIGINAL PAPER

Changes in activities of antioxidant enzymes and their gene expression during leaf development of sweetpotato

Yun-Hee Kim · Cha Young Kim · Haeng-Soon Lee · Sang-Soo Kwak

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Abstract To understand the functions of antioxidant enzymes during leaf development in sweetpotato, we investigated the activities of several antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APX) and catalase (CAT). Significant increases were observed in the activities of SOD, POX and APX during the late stage of leaf development, whereas CAT activity increased during the early developmental stage. By RT-PCR analysis, various POX and APX genes showed differential expression patterns during leaf development. Four POX genes *swpa3, swpa4, swpa6, swpb4* and one APX gene *swAPX1* exhibited high levels of gene expression during the senescence stage of leaf development, but two POX genes, *swpa1* and *swpa7* were preferentially expressed at both the mature green and the late senescence stages of leaf development. These results indicate that hydrogen peroxide (H_2O_2)-related antioxidant enzymes are differentially regulated in the process of leaf development of sweetpotato.

Keywords Antioxidant enzyme · Leaf development · Senescence · Sweetpotato

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
Chl	Chlorophyll
Fv/Fm	Current photochemical capacity of PSII
NBT	Nitro blue tetrazolium
POX	Peroxidase
ROS	Reactive oxygen species
SAG	Senescence-associated gene
SOD	Superoxide dismutase

Y.-H. Kim · C. Y. Kim · H.-S. Lee · S.-S. Kwak (✉)
Environmental Biotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Oun-dong 52, Yusong-gu, Daejeon 305-806, Republic of Korea
e-mail: sskwak@kribb.re.kr

Introduction

Leaf senescence is the final stage of leaf development that is controlled by both external and internal factors. Internal factors include age and phytohormone levels; external factors include stresses such as extreme temperature, shading, high light, drought, nutrient deficiency and pathogen infection (Munne-Bosch and Alegre 2002; Erwin and Renate 2003). It is typified by loss of chlorophyll (Chl) followed by degradation of proteins, nucleic acids, and lipids, and is associated with increased expression of enzymes that hydrolyze these macromolecules (Munne-Bosch and Alegre 2002; Yoshida 2003). Leaf senescence is also regulated by the coordinated expression of specific genes, and many senescence-associated genes (SAGs) have been identified in senescing leaves (Buchanan-Wollaston 1997; Quirino et al. 2000). The senescence process has been shown to lead to the production of reactive oxygen species (ROS) and the cell death by over-accumulation of ROS in plants (Asada 1999; Dat 2000; Prochazkova and Wilhelmova 2007). Leaf senescence as part of plant development can be influenced by the environmental conditions. Plants usually respond to various environmental conditions by triggering various defense mechanisms; one such response is the antioxidant defense system, including antioxidant enzymes (Asada 1999; Noctor and Foyer 1998). SOD converts superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2). POX reduces H_2O_2 to water

using various substrates as electron donors, and APX uses ascorbate to reduce H_2O_2 to water. CAT can break it down into water and oxygen. In the presence of O_2^- and H_2O_2, trace amounts of transition metals can give rise to the highly toxic hydroxyl radical ($OH^·$). Rapid detoxification of both O_2^- and H_2O_2 is essential for preventing oxidative damage. Furthermore, various antioxidant enzyme activities were changed during leaf development in various plant species, such as arabidopsis (Abarca et al. 2001), maize (Prochazkova et al. 2001), pea (del Rio et al. 2003), *Ramonda serbica* (Veljovic-jovanovic et al. 2006), ginkgo and birch (Kukavica and Jovanovic 2004). In particular, the induction of specific isoenzymes of POXs in many plants has been reported as being among the biomarkers of various environmental stresses and leaf senescence (Castillo 1992; Passardi et al. 2005; Cosio and Dunand 2009).

In our previous studies, ten POX cDNAs and one APX cDNA clones were isolated from the culture cells of sweetpotato (Huh et al. 1997; Kim et al. 1999; Park et al. 2003, 2004). Additionally, three POX cDNAs were also isolated from dehydrated fibrous roots of sweetpotato (Kim et al. 2008). Expression profiles of 14 antioxidant genes were performed as the first step toward understanding the physiological functions of each gene in response to various environmental stresses such as wounding, chilling, dehydration, stress-related chemicals and pathogen infection (Huh et al. 1997; Kim et al. 1999, 2000, 2007, 2008; Park et al. 2003, 2004; Jang et al. 2004). However, little is known about expression patterns of POXs and APX genes during leaf development of sweetpotato. Therefore, in this study, we describe the changes in activities and gene expression patterns of various antioxidant enzymes during leaf development of sweetpotato.

Materials and methods

Plant material

Sweetpotato (*Ipomoea batatas* (L.) Lam. cv. White star) plants were grown in the greenhouse for 3 months and the second to eighteenth leaves from shoot apical meristem were used for investigation. The intact leaf tissues were used for analysis of PSII photosynthetic efficiency and chlorophyll contents, enzyme activities and RT-PCR were determined using the leaf tissues collected in liquid nitrogen.

Analysis of photosynthetic activity

Photosynthetic activity from the leaves was estimated by chlorophyll fluorescence determination of photochemical yield (*Fv*/*Fm*), which represents the maximal yield of the photochemical reaction on photosystem II (PSII), using a portable chlorophyll fluorescence meter (Handy PEA, Hansatech, England).

Analysis of Chl content

Measurement of Chl content was performed with 0.1 g of fresh weight leaf materials which was quickly frozen in liquid nitrogen and then extracted with 2 ml of methanol. Samples were centrifuged at 12,000*g* for 15 min at 4°C and the Chl content in the supernatant was analyzed using a spectrophotometer according to the method of Porra et al. (1989).

Determination of total protein contents and antioxidant enzyme activities

The leaves of sweetpotato were homogenized on ice with a mortar in a 0.1 M potassium phosphate buffer (pH 7.0). Protein concentration was determined according to the Bradford (1976) method using Bio-Rad protein assay reagent. SOD activity was measured according to the method of McCord and Fridovich (1969) using xanthine, xanthine oxidase, and cytochrome *c*. One unit of SOD was defined as the amount of enzyme that inhibits the rate of ferric-cytochrome *c* reduction by 50%. POX activity was assayed according to the method described by Kwak et al. (1995) using pyrogallol as a substrate. One unit of POX activity was defined as the amount of enzyme that forming 1 mg of purpurogallin from pyrogallol during 20 s at 420 nm. APX activity was assayed according to the method described by Nakano and Asada (1981) using ascorbic acid as a substrate. The oxidation of ascorbate was initiated by H_2O_2, and the decrease at 290 nm for 1.5 min was monitored. One unit of APX was defined as the amount of enzyme oxidizing 1 μM of ascorbate. CAT activity was assayed according to the method described by Aebi (1984). The activity was determined by the decrease at 240 nm for 1 min due to H_2O_2 consumption.

Native poly-acrylamide gel electrophoresis (PAGE)

Native PAGE of SOD and POX were performed on a 7.5% gel at 120 V at 4°C (Beauchamp and Fridovich 1971). For SOD activity staining, the gel was incubated in the dark for 30 min in staining buffer [50 mM potassium phosphate buffer, pH 7.8, 0.026 mM riboflavin, 0.25 mM nitro blue tetrazolium (NBT), 0.2% TEMED] and then exposed to a light box until the SOD activity bands became visible. The SOD isozymes were differentiated by incubating the gel for 20 min in 50 mM potassium phosphate buffer (pH 7.8) containing either 3 mM KCN or 5 mM H_2O_2 before staining for activity. CuZnSODs were inhibited by KCN

and H_2O_2; FeSODs were resistant to KCN but were inactivated by H_2O_2; MnSODs were resistant to both inhibitors (Beauchamp and Fridovich 1971). POX gel assays were conducted according to Kim et al. (1994) with slightly modifications. After electrophoresis, the gel was stained with 1% benzidine and 1.5% H_2O_2.

RT-PCR analysis

Total RNA was extracted from the leaves of sweetpotato at different positions using the Trizol reagent (Invitrogen, USA). Fisrt-strand cDNA was synthesized from total RNA (1 μg) using the Improm-II™ reverse transcription kit (Promega, USA) according to the manufacturer's instruction. PCR amplification reactions were initially incubated at 94°C for 5 min, followed by 25–30 cycles at 94°C for 30 s, 42–58°C (depending on melting temperature of each gene-specific primer) for 30 s, and 72°C for 45 s. As an internal control of reverse transcription, 18S internal standards (Ambion, USA) were used. Gene-specific primers for PCR were designed from the 3′-UTRs or the region near the translation stop codon of each gene. Primer sets of the POXs and APX genes were used as described by previous reports (Park et al. 2003, 2004; Kim et al. 2007, 2008). Primer sets of sweetpotato SAGs are as follows; *spa15* primer set (Forward: 5′-GAGTTCTCCCGATGTC AATA-3′, reverse: 5′-CTTCTGGTGATTTTTCGTTC-3′). *Spg31* primer pair (Forward: 5′-TGGGAGTAAGTATTGG TTGG-3′, reverse: 5′-CAGAGGCGTTTTAGGAATAA-3′).

Statistical analysis

Data were statistically analyzed with Statistical Package for the Social Sciences (SPSS 12). Means were separated using Duncan's multiple range test at $P = 0.05$.

Results

Reduction of total proteins, photosynthetic efficiency and Chl contents

To investigate physiological changes during leaf development in sweetpotato, total proteins, PSII photosynthetic efficiency and Chl contents were analyzed using the leaves of 3-month-old plants. The leaves of sweetpotato remained green from the second to the tenth position, while gradual chlorosis was observed from the twelfth sweetpotato leaves (Fig. 1). Total protein contents and photosynthetic efficiency were measured in the leaves at different positions on the shoot apical meristem (Figs. 2a, b). Total proteins and photosynthetic efficiency reduced to 54 and 39% at the eighteenth leaf compared to that of the second leaf,

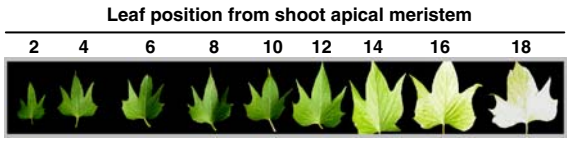

Fig. 1 Position-dependent leaf development and senescence symptom in sweetpotato. *Photographs* show representative leaves at each position from shoot apical meristem. Sweetpotato was grown in a green house for 3 months

respectively, whereas no significant differences were observed in the second to tenth leaf, which all had only about 12% decrease. Total Chl contents slightly decreased in the tenth and twelfth leaves, whereas they decreased about 40–80% in the fourteenth to eighteenth ones (Fig. 2c). The progression of leaf development was determined by comparing the Chl content of experimental leaves to that of fully expanded mature green leaves by modified the method of Lichtenthaler (1987). The various stages of leaf development were designated as: mature green stage, Chl content 90–100% (second to eighth); early senescence stage, 80–90% (tenth to twelfth); senescence stage, 40–80% (fourteenth to sixteenth); late senescence stage, 40–10% (eighteenth). These results indicate that physiological changes during leaf development in sweetpotato appear from the early senescence stage (tenth to twelfth leaves) by reduction of total proteins, photosynthetic rate and Chl contents.

Changes in activities of various antioxidant enzymes

To examine whether the H_2O_2-related antioxidant enzymes are involved in the process of leaf development in sweetpotato, we measured the activities of SOD, POX, APX and CAT. SOD activity gradually increased from the second until the sixteenth leaf positions. The highest level was detected in the sixteenth leaf with a 2.7-fold increase compared to that of the second leaf, and its activity decreased in the eighteenth leaf (Fig. 3a). In contrast, POX activity remained constant from the second to the fourteenth leaf positions, and its activity increased at the sixteenth and eighteenth positions. The eighteenth leaf showed the highest POX activity with a 2.5-fold increase compared to that of the second leaf (Fig. 3b). In case of APX activity, it greatly increased at the positions the fourteenth to sixteenth. The highest level was observed in the sixteenth leaf with a 6.6-fold increase compared to that of the second leaf (Fig. 3c). CAT activity displayed a gradual increasing pattern from the second to the eighth and then kept constant until the fourteenth position, showing a maximum in the eighth to fourteenth leaf with a 3.6-fold increase compared to that of the second leaf. The activity greatly decreased at the leaf positions the sixteenth

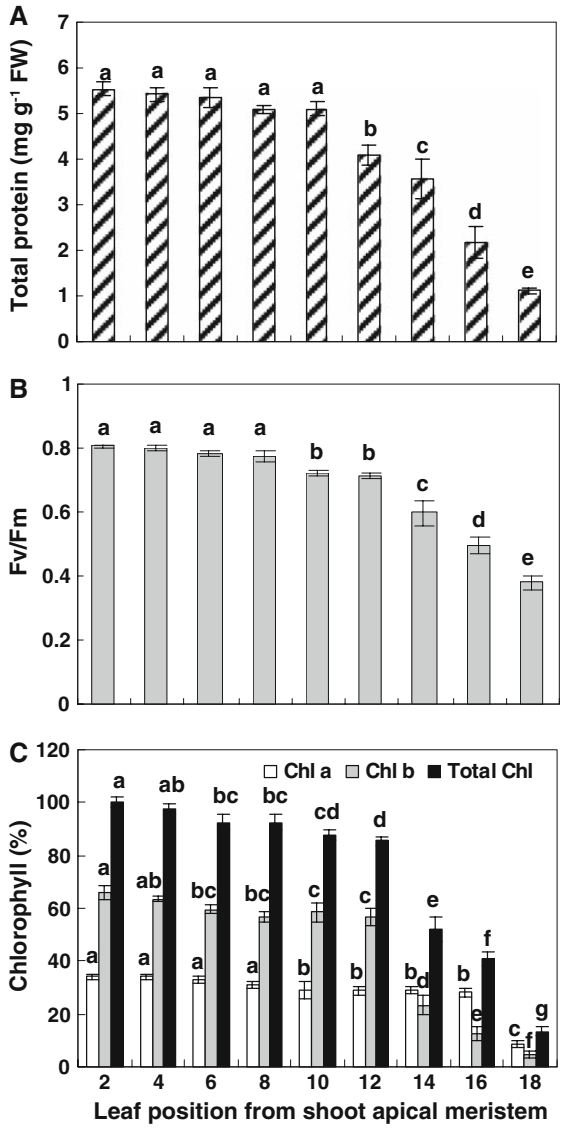

Fig. 2 Physiological changes during leaf development in sweetpotato. Changes in total protein contents **A**, PSII photosynthesis efficiency **B** and Chl contents **C** during leaf development in sweetpotato. Data mean ± SE of three independent plant leaves. *Bars* carrying the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test

and the eighteenth (Fig. 3d). In addition, we investigated the antioxidant enzyme activity during leaf development using native gels. Among the SOD isoenzymes, CuZnSOD and MnSOD were detected but not FeSOD (Fig. 4a). In particular, MnSOD isozymes showed a gradual increasing pattern over the leaf development with a high activity from the twelfth to the eighteenth leaf positions. Many POX isoenzyme bands increased during leaf development. Interestingly, POX isoenzymes are induced as shown at the bottom of native gel in the eighteenth leaf (Fig. 4b). Isoenzyme patterns of APX and CAT in native gel assay were similar to the changes in these enzymes activities shown in Fig. 3 (data not shown). These results suggest that CAT enzymes mainly function at the leaf positions from the eighth to fourteenth with a low activity during the late stage of leaf development, whereas other antioxidant enzymes such as SOD, APX and POX are active at the late stage of leaf development in sweetpotato.

Differential expression of POXs and APX genes

Expression profiles of thirteen POXs and one APX gene subjected to leaf development in sweetpotato were studied by RT-PCR with gene-specific primers. Two senescence marker genes, *spa15* and *spg31*, in sweetpotato were used as positive controls (Huang et al. 2001; Yap et al. 2003). As shown in Fig. 5, *swpa3, swpa4, swpa6, swpb4* and *swAPX1* showed high levels of expression at the senescence or late senescence stages, whereas *swpa1* and *swpa7* were expressed in leaves at both the mature green (the second leaf position) and the late senescence stages (the eighteenth leaf position). However, the transcripts of the other POX genes such as *swpa2, swpa5, swpb1, swpb2, swpb3, swpb5* and *swpn1* were not detected at any stages of leaf development (data not shown). As the previous report, in the tenth leaf to the sixteenth leaf, the transcript expression of *spa15* increased and the chlorophyll contents decreased, whereas the expression of *spg31* was induced in the sixteenth and eighteenth leaf. Interestingly, antioxidant genes in sweetpotato were expressed at the late senescence stage as well as the mature green stage, suggesting that these genes are differentially regulated during the early or late stages of leaf development.

Discussion

In this study, we evaluated the changes in antioxidant enzyme activities and expression profiles of various antioxidant genes during sweetpotato leaf development. The activities of SOD, APX and POX increased in the late development process, whereas CAT activity increased during the early leaf development of sweetpotato. Antioxidant genes were also differentially expressed at the early or the late leaf development stage of sweetpotato.

It has been suggested that ROS are involved in leaf senescence and stress responses (Zimmermann and Zentgraf 2005). ROS accumulation during senescence has been shown to accompany programmed cell death, implicating the change of cellular antioxidant defense. We showed that SOD activity increased in the leaves of sweetpotato undergoing senescing process (Fig. 3). The increase of SOD activity has been proposed to be a protective mechanism that it contributes to delaying the

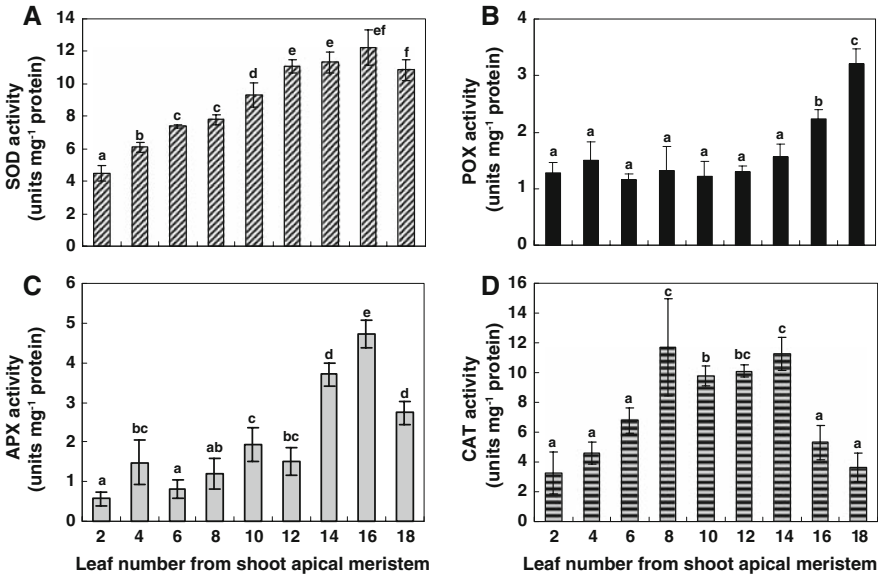

Fig. 3 Measurement of antioxidant enzyme activities during leaf development in sweetpotato. Changes in enzyme activities of SOD **A**, POX **B**, APX **C** and CAT **D**, Data mean ± SE of three independent plant leaves. *Bars* carrying the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test

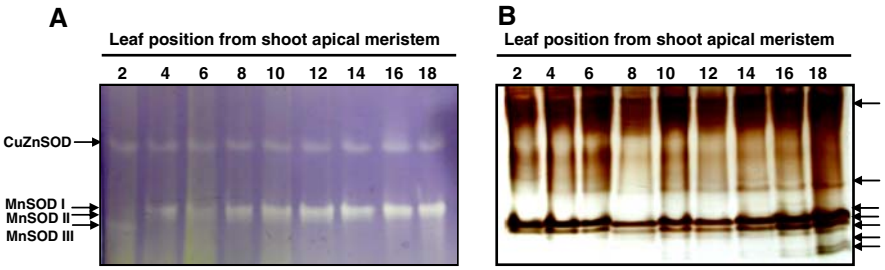

Fig. 4 Native gel analysis of SOD and POX isozymes during leaf development. SOD activity was detected with a negative staining solution using NBT after electrophoresis **A**. Seventy micrograms of total protein concentrated from leaves were loaded in each line. POX activity was detected with a positive staining solution using benzidine after electrophoresis **B**. Forty micrograms of total protein concentrated from leaves were loaded in each line. *Arrows* indicates major isoenzyme bands of SOD and POX

senescence process by O_2^- detoxification. Interestingly, the activities of MnSOD isoenzymes greatly increased (Fig. 4). The result is consistent with that MnSOD being the major isoform responsible for O_2^- scavenging during senescence in mitochondria (del Rio et al. 2003). Increasing evidence also suggests that MnSOD activity increases during leaf senescence in various plant species, such as maize (Prochazkova et al. 2001), pea (del Rio et al. 2003), *Ramonda serbica* (Veljovic-jovanovic et al. 2006), ginkgo and birch (Kukavica and Jovanovic 2004). Therefore, it is likely that MnSOD plays major roles in O_2^- scavenging and H_2O_2 production during leaf senescence in sweetpotato.

POX, APX and CAT are the major enzymes responsible for H_2O_2 scavenging during oxidative stress. Our results have shown that an increase of POX and APX activity in the leaves of sweetpotato coincided with a decrease in CAT activity (Fig. 3), indicating that the leaf antioxidant defense system could keep H_2O_2 at a low level and maintain redox homeostasis. It was shown that in senescing leaves POX replaces CAT in autumn yellowing of gingko and birch plants (Kukavica and Jovanovic 2004). Consistently, high activities of POX and APX were detected but CAT activity was low in leaves at the late developmental stage (Fig. 2, 3), suggesting that POX and APX play major roles in H_2O_2 scavenging during later stages of senescence. CAT was shown to be generally abundant in peroxisomes of green leaves in plant (del Rio et al. 1998). Similarly, we also observed comparatively high CAT activity in the early leaf development stage (Fig. 3). It has been reported that increase of H_2O_2 content is involved in the reduction of CAT activity in peroxisomes during leaf senescence (del Rio et al. 1998). Therefore, our results suggest that artificially up regulating the levels of antioxidant enzymes may delay leaf senescence that may, under some circumstances, increase yield.

It has been reported that expression of photosynthesis-associated genes decreased and *SAGs* increased during leaf development in various plant species (Buchanan-Wollaston et al. 2003). The *spa15,* a sweetpotato *SAG* gene, was induced during leaf senescence when leaf Chl contents decreased by 10–60% and the sweetpotato *SAG* gene, *spg31* was up-regulated during the late senescence stage

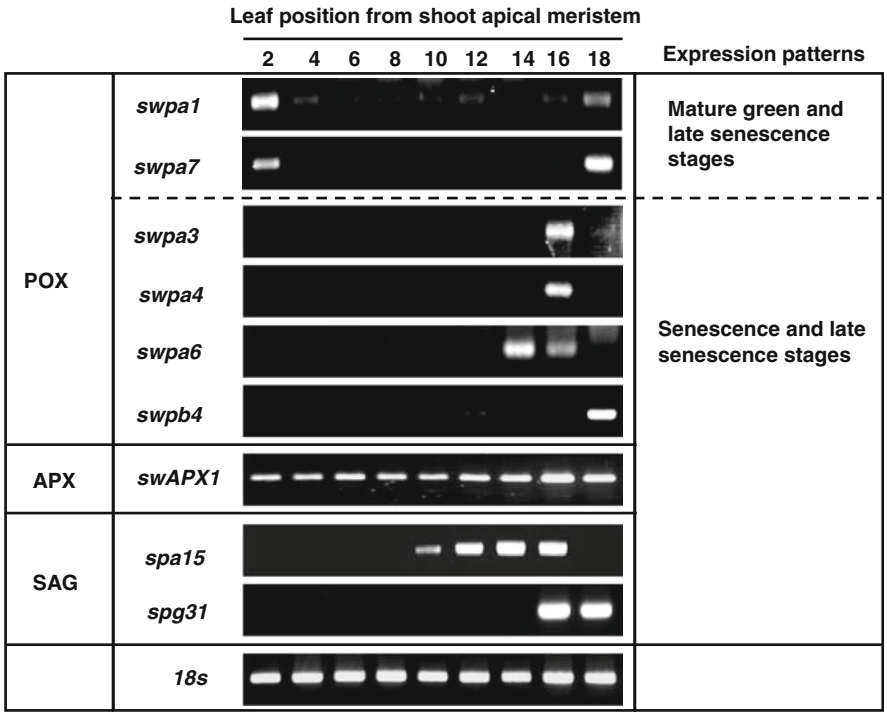

Fig. 5 RT-PCR analysis of antioxidant enzyme genes during leaf development of sweetpotato. Two sweetpotato *SAGs*, *spa15* and *spg31*, were used as senescence markers. 18S RNA was used as a control for equal loading

when its contents reduced by over 60% (Fig. 5). We previously reported the expression profiles of thirteen POXs and one APX genes in sweetpotato in response to various abiotic stresses and pathogenic infection (Huh et al. 1997; Kim et al. 1999, 2000, 2007, 2008 Park et al. 2003, 2004; Jang et al. 2004). As observed in our restricted responses using limited growth conditions and time during plant development, some antioxidant genes displayed a stage-specific response, including the late leaf development stage, such as senescence and late senescence. Four POXs including *swpa3, swpa4, swpa6,* and *swpb4,* and one *swAPX1* were inducible at the late developmental stage (the fourteenth—the eighteenth leaves) (Fig. 5). These results indicate that they may play a major role in H_2O_2 scavenging during the late leaf senescence in the sweetpotato plant.

In conclusion, we showed the changes in antioxidant enzyme activities and expression profiles of various antioxidant genes during sweetpotato leaf development. Diverse expression patterns of antioxidant genes will provide information on the possible functions of each gene during leaf development of sweetpotato. Changes in antioxidant enzyme levels during leaf development under various abiotic and biotic stresses remain to be studied. Therefore, further study of antioxidant mechanism in leaves of different developmental stages will be interesting in terms of environmental stress.

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