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ORIGINAL PAPER

Diverse antioxidant enzyme levels in different sweetpotato root types during storage root formation

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Abstract Sweetpotato forms different types of root during storage root production, including fibrous roots (FR), thick roots (TR), and storage roots (SR). To understand the functions that antioxidant enzymes play in the development of these different roots, we investigated the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), and polyphenol oxidase (PPO). Significantly higher activity levels were observed in FR than in both TR and SR for SOD, APX and CAT. Both POD and PPO activity were significantly higher in FR and TR than in SR. Quantitative RT-PCR analysis was used to investigate antioxidant gene expression patterns in the different stage and root types of sweetpotato. Some genes displayed root-type or stage specific responses depending on the root part. *CuZn-SOD*, *APX*, *swpa7*, *swpa8*, *swb6*, and *swpb7* were expressed at significantly higher levels in the FR and SR than in the TR. *CAT*, *swpa9*, *swpa4*, and *swpb7* exhibited significantly higher expression levels in the FR and TR than in the SR. In addition, they also showed higher levels of expression in the lower parts of the FR and TR at the early stage, whereas SR was expressed in the upper part. These results indicate that antioxidant enzymes are differentially regulated during the early stages of sweetpotato root development.

Keywords Antioxidant enzyme · Different root development · Sweetpotato

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
FR	Fibrous roots
GPX	Glutathione peroxidase
GST	Glutathione-*S*-transferase
POD	Peroxidase
PPO	Polyphenol oxidase
ROS	Reactive oxygen species
SR	Storage roots
TR	Thick roots
TRX	Thioredoxin

Introduction

Sweetpotato [*Ipomoea batatas* (L.) Lam.] ranks seventh in annual production among global food crops. It is not only energy-rich, with its high complex carbohydrate and sugar content, but is also a good source of nutrients and natural antioxidant compounds such as vitamins, polyphenols, anthocyanins, and carotenoids (Yoshinaga et al. 1999; Teow et al. 2007). Sweetpotato is also a relatively stress-tolerant crop and can be grown on marginal land, making it a globally important subsistence food source. However, the

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molecular and biochemical mechanisms underlying root stress tolerance are not well understood.

Sweetpotato yield and quality is dependent upon storage root (SR) formation during root development. Three different sweetpotato roots have been identified on the basis of anatomical studies (Wilson and Lowe 1973; Tanaka et al. 2005; Noh et al. 2010). The sweetpotato plant initially produces white fibrous roots (FR). Some FR subsequently acquire pigmentation and undergo lignified thickening growth to form thick roots (TR), and may ultimately develop into SR. The accumulation mechanisms of some storage compounds, such as sporamin and amylase, have been determined (Hattori et al. 1990; Ohta et al. 1991). Recent transcriptional studies evaluated gene expression during early SR development (Firon et al. 2013). Firon et al. (2013) found that starch biosynthesis-related genes were up-regulated and lignin biosynthesis-related genes were down-regulated during early SR formation. However, despite these prior root development-related studies, the molecular and biochemical mechanisms underlying development of the different sweetpotato roots during SR formation remain unclear.

Reactive oxygen species (ROS), such as superoxide anionic radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), are normal by-products of aerobic cell metabolism, but ROS levels can increase during environmental stress. Overaccumulation of ROS can lead to cell death, and so levels must be tightly controlled (Asada 1999; Dat 2000). Plants utilize antioxidant defense systems to protect them against toxic ROS, and plant stress-resistance correlates with capacity to scavenge or detoxify ROS. Stress conditions stimulate changes in antioxidant enzyme activity levels in leaves or roots of various plant species (de Azevedo Neto et al. 2006; Zhang et al. 2007; Wang et al. 2009), but the root antioxidant defense mechanism has not been determined in sweetpotato.

Previously, we demonstrated changes in antioxidant enzyme activities during sweetpotato leaf development (Kim et al. 2009). We also used dehydration-treated sweetpotato FR under various abiotic stress conditions to isolate and characterize genes that encode intracellular and extracellular antioxidants (Kim et al. 2008, 2013). However, little is known about antioxidant changes in the different sweetpotato roots during SR formation. In this study, we describe the changes in gene expression and enzyme activity of various antioxidants in sweetpotato roots during SR formation.

Materials and methods

Plant materials

Sweetpotato [*Ipomoea batatas* (L.) Lam. cv. Yulmi] plants were grown in a greenhouse for 4 months and FR, TR, and SR were separated. Intact root tissues were used for analysis of lignin, total phenolics, and enzyme activities, and liquid nitrogen preserved samples were used for quantitative RT-PCR.

Determination of total phenolics and lignin contents

Soluble phenolics were extracted and determined in accordance with the methods established by Stadnik and Buchenauer (2000). Methanol extracts were centrifuged for 10 min at 12,000*g* and the supernatants used for a Folin–Ciocalteau assay. Soluble phenolics were determined spectroscopically at 725 nm using *p*-coumaric acid as a standard. The remaining root tissue was dried for 48 h at 60 °C, powdered, and used for quantification of lignin content via thioglycolic acid assay (Stadnik and Buchenauer 2000; Hatfield and Fukushima 2005). Briefly, 50 mg of dry powdered samples were treated with 0.5 mL of a 1:10 mixture of thioglycolic acid and 2 N HCl for 4 h at 100 °C. After two subsequent water washes, the lignothioglycolic acid was extracted from the pellet with 1 mL of 0.5 N NaOH for 18 h. Lignin contents were determined at 280 nm using a lignin standard (alkali, 2-hydroxypropyl ether, Aldrich).

Enzyme activity assays

Total soluble protein was extracted from the different roots of sweetpotato plants using an extraction buffer and the concentration determined using the Bio-Rad protein assay (Bradford 1976). Extracted protein was used for POD, PPO, SOD, APX and CAT activity determination. POD activity was assayed according to the method described by Kwak et al. (1995) using pyrogallol as a substrate. One unit of POD activity was defined as the amount of enzyme required to form 1 mg of purpurogallin from pyrogallol in 20 s, as measured by absorbance at 420 nm. PPO activity was assayed according to the method described by Aquino-Bolanos and Mercado-Silva (2004) using catechol as a substrate. PPO activity was expressed as units of activity, where one unit of PPO was defined as the change in one unit of absorbance per second at 420 nm. SOD activity was assayed using the photochemical nitro blue tetrazolium (NBT) method (Beyer and Fridovich 1987). Photo-reduction of NBT (formation of purple formazan) was measured at 560 nm, and an inhibition curve was constructed against different volumes of extract. One unit of SOD is defined as being present in the volume of extract that causes inhibition of the photo-reduction of NBT by 50 %. APX activity was assayed according to the method of Nakano and Asada (1981) using ascorbic acid as a substrate. Ascorbate oxidation was initiated by H_2O_2 and the decrease in absorbance at 290 nm was monitored for 1 min 30 s. One unit of

APX was defined as the amount of enzyme oxidizing 1 mol of ascorbate per minute. CAT activity was assayed according to the method described by Aebi (1984). Activity was determined by the decrease in absorbance at 240 nm for 1 min due to H_2O_2 consumption.

Native poly-acrylamide gel electrophoresis (PAGE)

Native PAGE of POD, PPO, SOD, APX, and CAT was performed on a 7.5 % gel at 120 V at 4 °C (Beauchamp and Fridovich 1971). POD gel assays were conducted according to Kim et al. (1994) with slight modifications. After electrophoresis, the gel was stained with 1 % benzidine and 1.5 % H_2O_2. PPO gel assays were conducted according to Ayaz et al. (2008) with slight modifications. After electrophoresis, the gel was stained in 24 mM L-3,4-dihydroxyphenylalanine (L-DOPA) for PPO activity. 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 NBT, 0.2 % TEMED] and then exposed to a light box until the SOD activity bands became visible. SOD isoenzymes 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, and MnSODs were resistant to both inhibitors (Beauchamp and Fridovich 1971). APX isozymes were detected by equilibrating the gels in a solution composed of 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 min, and then 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. After washing with 50 mM sodium phosphate buffer (pH 7.0) for 1 min, gels were submerged in a solution of 50 mM sodium phosphate buffer (pH 7.8) with 28 mM TEMED and 1.25 mM NBT for 10 min at room temperature (Mittler and Zilinskas 1994). CAT isozymes were detected by immersing gels in a solution composed of 50 mM potassium phosphate buffer (pH 7.0) containing horseradish POD (50 μg ml^{-1}) for 45 min. H_2O_2 was then added to a concentration of 5 mM and gels were soaked for 10 min. Gels were then rapidly rinsed twice with water and were placed into 0.5 mg ml^{-1} of diamino benzidine (DAB) in phosphate buffer until staining was completed (Clar et al. 1984).

Gene expression analysis

Total RNA was isolated from sweetpotato roots using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated extensively with RNase-free DNase I to remove any contaminating genomic DNA. For quantitative expression analysis of various antioxidant genes, first strand cDNA was generated from total RNA (2 μg) using MMLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Quantitative RT-PCR was performed in a fluorometric thermal cycler (DNA Engine Opticon 2, MJ Research, Waltham, MA, USA) using EverGreen fluorescent dye according to the manufacturer's instructions. The experimental quality control comparisons of repeated samples were assessed using CT values between the three replicates. Samples with values $> 1.5 \times$ the differential values were removed from the data set. Gel electrophoresis confirmed the presence of a single PCR product of the correct size, and samples with multiple peaks in the dissociation graph were dismissed to exclude non-specific PCR reactions. Linear data were normalized to the mean CT of α-tubulin as a reference gene, and the relative expression ratio was calculated using the $2^{-\Delta\Delta C_t}$ method. The expression levels of sweetpotato POD genes were analyzed by quantitative RT-PCR using previously described gene-specific primers (Kim et al. 2008, 2013).

Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA). The subsequent multiple comparisons were performed using Duncan's multiple range test. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 12). Statistical significance was set at $P < 0.05$.

Results

Changes in levels of lignin, phenolic, and related enzymes

To investigate the biochemical changes that occur during SR formation in the different sweetpotato roots, we first classified the adventitious roots into three types according to previous categories: FR, TR and SR (Wilson and Lowe 1973; Tanaka et al. 2005) (Fig. 1).

To examine whether the lignification-related biochemical changes are involved in the process of differential root development of sweetpotato, we measured total lignin and phenolic contents (Fig. 2). As expected, SR showed the lowest total levels of lignin and phenolics. Lignin and phenolic levels were highest in TR, at 1.9 and 1.8 fold levels in FR, respectively, and 21.6 and 11.3 fold levels in SR, respectively. Additionally, to examine whether lignin and phenolic accumulation-related enzymes are involved in the process of lignification and phenolic accumulation during root development, we investigated peroxidase (POD) and polyphenol oxidase (PPO) activities. POD

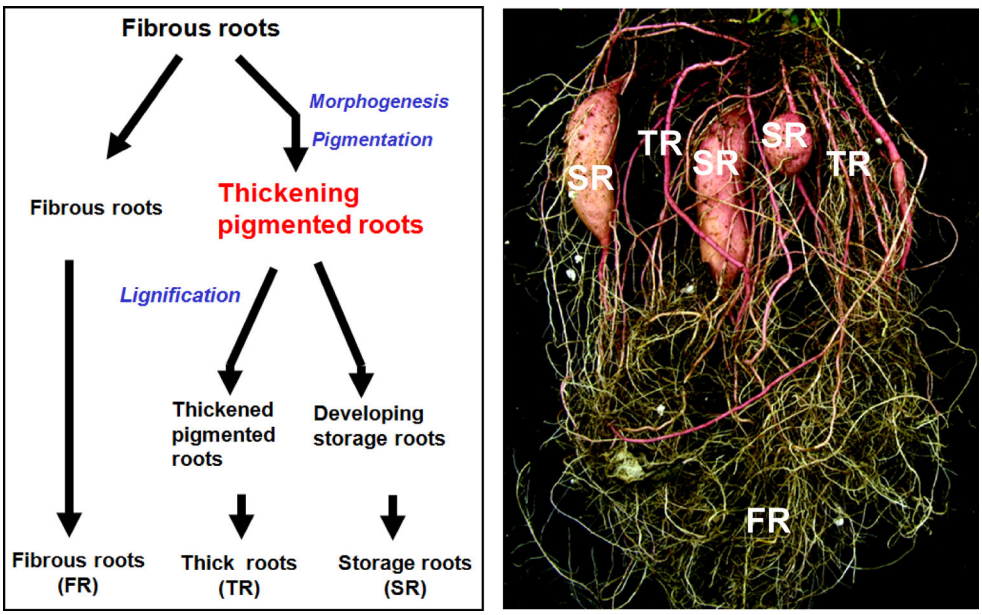

Fig. 1 Sweetpotato root development. Photographs shows representative sweetpotato root types after greenhouse growth for 4 months. *FR* fibrous roots, *TR* thick roots, *SR* storage roots

activity corresponded to the total lignin content in the different sweetpotato roots (Fig. 2c). POD activity was lowest in SR, with levels 10.3 and 6.3 folds lower than in TR and FR, respectively. PPO activities were 3.68 U/mg protein (FR), 2.16 U/mg (TR), and 1.16 U/mg (SR) (Fig. 2d). Relative POD and PPO isoenzyme activities in the different roots were determined by native gel assay and displayed similar patterns to the total enzyme activities.

Changes of ROS scavenging enzymes

To determine whether H_2O_2-related antioxidant enzymes are involved in sweetpotato differential root development, we measured the SOD, APX, and CAT activities. The highest SOD activities were detected in FR, with levels 1.9 and 1.66 folds higher than those in TR and SR, respectively (Fig. 3). Native gel analysis was used to distinguish between isoenzymes, and detected CuZnSOD and MnSOD in sweetpotato roots, but not FeSOD. Higher CuZnSOD and MnSOD activities were detected in FR compared with TR and SR.

As observed for SOD, the highest APX and CAT activities were detected in FR (Fig. 4). APX and CAT levels were at their lowest in SR. Strong APX or CAT isoenzyme bands were detected in FR during different sweetpotato root development, and relative APX and CAT isoenzyme levels in the different roots corresponded to overall enzyme activities.

Differential expression of antioxidant genes

To investigate the molecular change that occur at different root development stages in sweetpotato, each root was divided into three parts, namely the upper part (UP), middle part (MP), and lower part (LP) (Fig. 5a). The expression profiles of intracellular and extracellular antioxidant genes at different root development stages in each sweetpotato root part were studied by performing quantitative RT-PCR with gene-specific primers (Fig. 5b). Six intracellular antioxidant genes were highly expressed in the FR. Among these, *CuZnSOD*, *APX*, *CAT*, and *TRX* genes were expressed in the TR and SR. Almost all intracellular genes showed high expression levels in the later stage in the LP of the FR, TR, and SR. Extracellular PODs showed high expression levels in the FR and TR. The genes *swpa7*, *swpa8*, *swb6*, and *swpb7* were also expressed in the SR. PODs were expressed at high levels in the LP of the FR and TR at the early stage, whereas they were expressed at high levels in the UP of the SR.

Discussion

Plant roots are the primary sensors of abiotic stresses such as high salinity and drought, and, as a result, root development is normally limited by changes in environmental conditions. Despite considerable efforts to investigate stress responses during root development, the molecular and biochemical mechanisms involved remain incompletely understood. In this study, we used sweetpotato to evaluate changes in antioxidant enzyme activities and antioxidant gene expression profiles in three root types: FR, TR, and storage roots (SR). Our results indicate that, of the three root types investigated during SR formation, antioxidant defense mechanisms were most prevalent in FR.

Sweetpotato plants produce thin, white FR in the early stages of root development that can then develop into

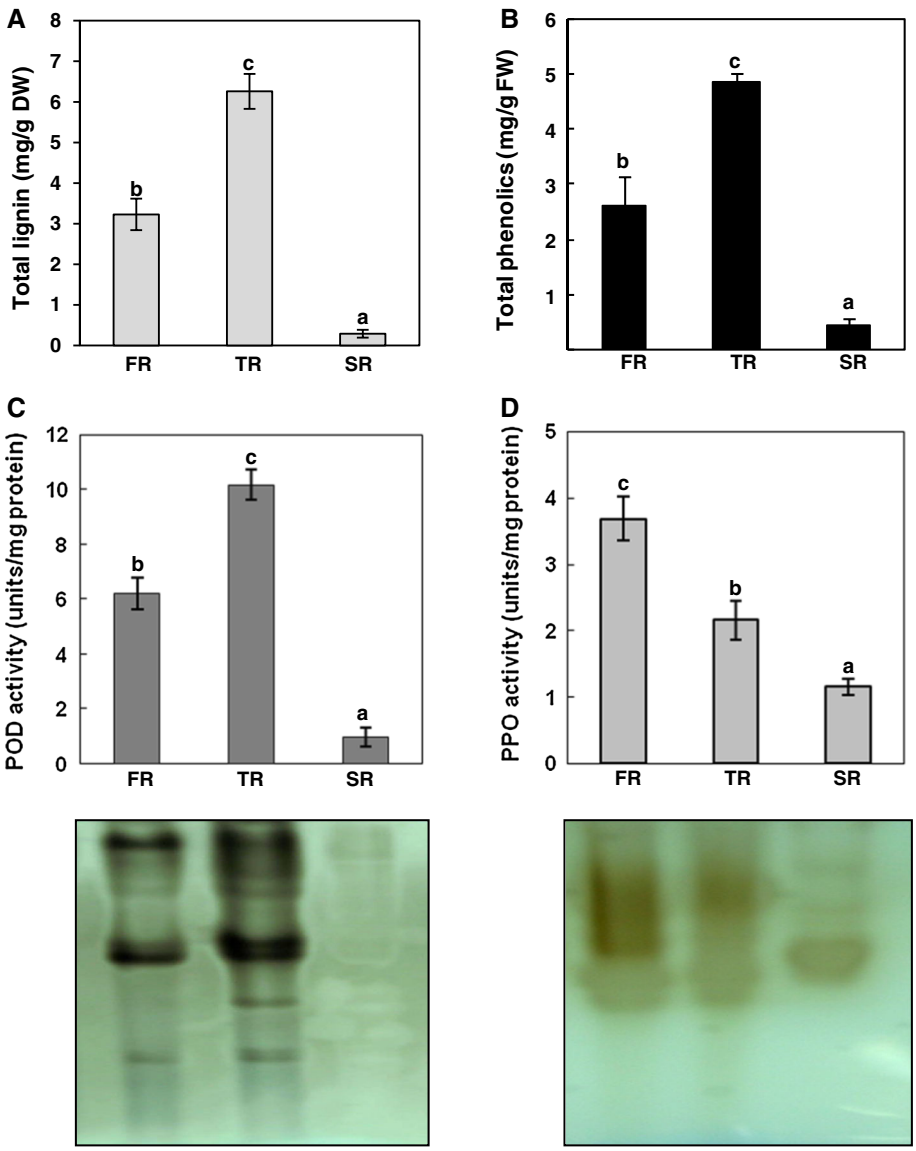

Fig. 2 Total lignin and phenolic levels and related enzyme activities in different sweetpotato roots. Total root lignin content **a.** Total root soluble phenolic content **b**. POD activity and isoenzyme patterns as determined by spectroscopy and native gel analysis, respectively **c**. PPO activity isoenzyme patterns as determined by spectroscopy and native gel analysis, respectively **d**. Data shown are the mean ± SE of five independent plants. *Bars* labeled with the same letter are not significantly different ($P \geq 0.05$) according to Duncan's multiple range test. Bands on gel images (*bottom* of figure) indicate the major POD and PPO isoenzymes

mature FR or different root types such as TR and SR (Wilson and Lowe 1973; Tanaka et al. 2005; Noh et al. 2010). TR are generated from white FR by pigmentation and lignification. FR can become further pigmented and swell, finally developing into SR. TR and SR thus have different biochemical features, such as lignification in TR development and starch accumulation during SR formation. Firon et al. (2013) demonstrated that starch biosynthesis-related gene expression increased, lignin biosynthesis-related gene expression decreased, and cell wall POD gene expression decreased during early SR formation in sweetpotato. In this study, sweetpotato plants exhibited differential accumulation of total lignin and phenolics in the different root tissues, with TR and FR having higher lignin and phenolic accumulation levels than SR (Fig. 2).

Lignin and phenolic-related activities were determined by POD and PPO activity assays, respectively (Fig. 2). POD activity levels were highest in TR, whereas PPO activity levels were highest in FR. Consistent with total lignin and phenolic contents, SR exhibited the lowest activity levels in both assays. PPOs add oxygen to a monophenol aromatic ring, producing an additional hydroxyl group, and then oxidize the diphenol to the corresponding quinine (Mayer 2006). Mechanical damage generally causes physical stress, which damages the plant tissue and alters the phenolic metabolism (Rhodes and Wooltorton 1978). Mechanical damage to plants thus causes surface browning as a result of increased phenolic content and activity of phenyl propanoid biosynthesis-related enzymes such as phenylalanine ammonia lyase (PAL) (Aquino-Bolanos et al. 2000). Plant browning

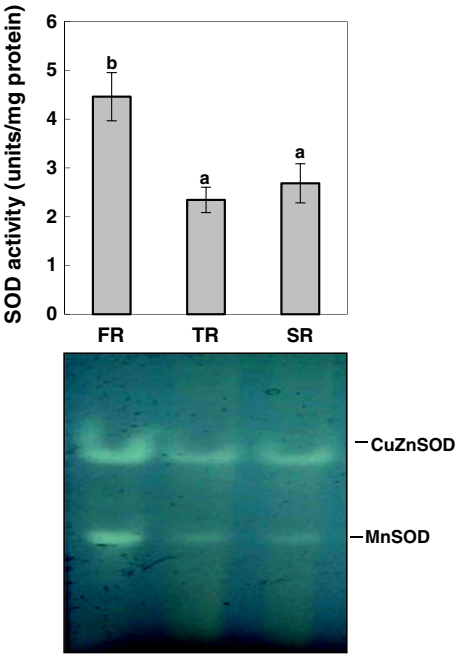

Fig. 3 SOD levels in different sweetpotato roots. SOD activity and isoenzyme patterns as determined by spectroscopy and native gel analysis, respectively. Data shown are the mean ± SE of five independent plants. *Bars* labeled with the same letter are not significantly different ($P \geq 0.05$), according to Duncan's multiple range test. On the gel image, *arrows* indicate bands containing the major SOD isoenzymes

induced by mechanical damage has been attributed to PPO activity on phenolic compounds, which causes their oxidation and polymerization and the consequent development of a brown color (Vamos-Vigyazo 1981; Mika et al. 2004). In addition, plants create physical barriers to counteract mechanical damage and limit tissue destruction; this response includes the synthesis of polyphenols such as lignin and suberin (Walter et al. 1990). Lignins defend against various pathogen and insect attacks by supporting and strengthening cell walls, facilitating water transport, and preventing degradation of cell wall polysaccharides (Hatfield and Vermerris 2001; Li et al. 2008). Plant PODs are involved in several plant metabolic processes such as auxin catabolism, various stress tolerance responses, the formation of bridges between cell wall components, and oxidation of cinnamyl alcohols before their polymerization during lignin and suberin formation (Quiroga et al. 2000; Passardi et al. 2004). PODs are involved in lignification at several levels, from monolignol polymerization at the cell wall level to mechanisms involving free radical generation (Whetten et al. 1998). Other enzymes, such as PPO and laccase, may also take part in the polymerization reactions (Richardson and McDougall 1997).

In the present study, we found that lignin levels, phenolic levels, and POD activity are highest in sweetpotato TR, whereas PPO activity is highest in FR (Fig. 2). This

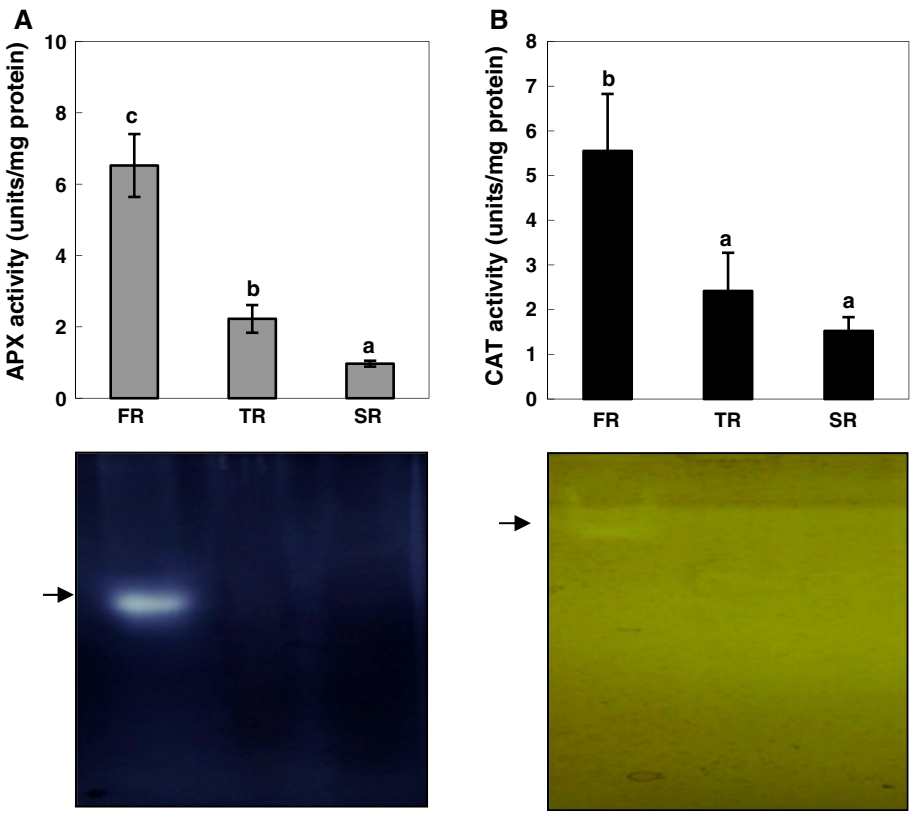

Fig. 4 APX and CAT levels activity in different sweetpotato roots. APX activity and isoenzyme patterns as determined by spectroscopy and native gel analysis, respectively **a**. CAT activity and isoenzyme patterns as determined by spectroscopy and native gel analysis, respectively **b**. Data shown are the mean ± SE of five independent roots. *Bars* labeled with the same letter are not significantly different ($P \geq 0.05$) according to Duncan's multiple range test. On the gel images, *arrows* indicate bands containing the major isoenzymes of CAT and APX

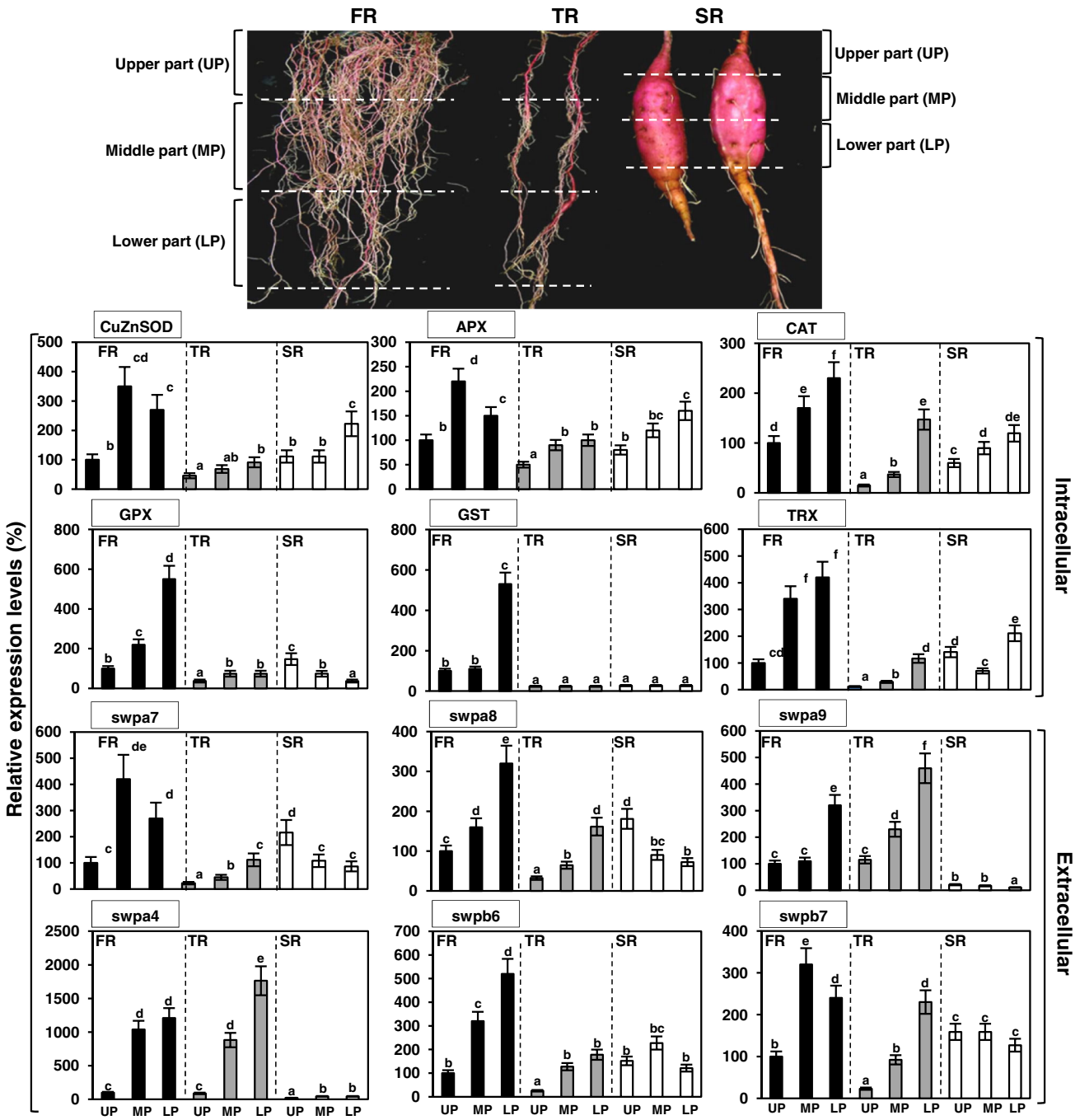

Fig. 5 Expression patterns of intracellular and extracellular antioxidant genes in different stage and root types of sweetpotato during roots development. **a** Classification of different root part during sweetpotato root development. Photographs shows representative sweetpotato root types after greenhouse growth for 4 months. *FR* fibrous roots, *TR* thick roots, *SR* storage roots, *UP* upper part, *MP* middle part, *LP* lower part. **b** Expression patterns of intracellular and extracellular antioxidant genes in different sweetpotato roots. Data shown are the mean ± SE of three independent roots. *Bars* labeled with the *same letter* are not significantly different ($P \geq 0.05$) according to Duncan's multiple range test

suggests that POD-dependent reactions are more important than PPO metabolism in the accumulation of lignin and phenolics in TR. Lignin, phenolics, and intermediaries produced by monolignol oxidation have been implicated in plant tissue browning in response to wound-induced physical injury (Aquino-Bolanos et al. 2000). PPO activity is thus unlikely to completely account for plant browning, suggesting that this also occurs as a consequence of

lignification. Our findings that total phenolic levels in FR and TR are higher than in SR and that PPO activity is highest in FR suggest a key role for PPO-dependent defense mechanisms on the accumulation of phenolics in the FR and TR (Fig. 2).

ROS are thought to be involved in development and stress responses in plants (Apel and Hirt 2004; Laloi et al. 2004). ROS accumulation during root development accompanies cell wall restructuring and root elongation, suggesting that regulation of cellular ROS levels by antioxidant systems allows both optimal root development and response to environmental changes. Within plant cells, SODs constitute the first line of defense against ROS, and their specialized functions might be due to the varied subcellular locations of the different enzymes (Alscher et al. 2002). When coupled with the necessary downstream events for full detoxification of toxic ROS levels in plants, SODs are among the most important aspects of the plant defense system. In this study, we found that SOD activity was higher in the FR than in both SR and TR (Fig. 3). High SOD activity levels are proposed to be part of a protective mechanism that delays senescence and aids development via O_2^- detoxification in the root parts. MnSOD and Cu-ZnSOD isoenzyme levels were also highest in the FR (Fig. 3), which is consistent with previous data indicating that MnSOD and CuZnSOD are the major isoforms responsible for O_2^- scavenging in sweetpotato leaves (Kim et al. 2009). Together, these data suggest that MnSOD and CuZnSOD play major roles in O_2^- detoxification during differential root development and leaf senescence.

APX and CAT are the major enzymes responsible for H_2O_2 scavenging during oxidative stress in plants (Shigeoka et al. 2002; Luna et al. 2004). Our results showed that the activity levels of both APX and CAT were highest in FR (Fig. 4), indicating that low H_2O_2 levels were maintained in FR through redox homeostasis involving enzymes such as SOD, APX and CAT. Therefore, our results suggest that artificially up-regulating antioxidant enzyme levels could improve root growth and development and may, under some circumstances, increase yield.

Previous research found that expression levels of sporamin and starch-biosynthesis-associated genes increase during SR formation in sweetpotato (Firon et al. 2013). We previously reported the expression profiles of intracellular antioxidant genes and extracellular POD genes in sweetpotato in response to various abiotic stress conditions, such as drought, salt, and low temperature (Kim et al. 2008, 2013). In the current study, we reexamined the activity of these genes at different developmental stages and in different root types. Some of the genes displayed root-type or stage specific responses (Fig. 5). The intracellular genes *CuZnSOD* and *APX,* and the extracellular genes *swpa7*, *swpa8*, *swb6,* and *swpb7* were expressed at significantly higher levels in the FR and SR than in the TR. Intracellular genes such as *CAT* and extracellular genes such as *swpa9*, *swpa4,* and *swpb7* exhibited significantly higher expression levels in the FR and TR than in the SR. The generally high levels of these genes in the FR indicate that they may have major defensive roles in H_2O_2 scavenging in the FR during differential root development in sweetpotato. In addition, intracellular and extracellular antioxidant genes showed high levels of expression at the early stage in the LP of the FR and TR, whereas SR was expressed in the UP. Thus, antioxidant genes were expressed at the early stage in the roots of sweetpotato, suggesting that these genes are differentially regulated during the early stages of development in the different parts of sweetpotato root.

In conclusion, we determined the activities of various antioxidant enzymes and their expression profiles during the differential root development stages of sweetpotato. Our results show that the activity and gene expression profiles of intracellular and extracellular antioxidant genes at different root stages in sweetpotato are regulated by root type and developmental stage. Intracellular antioxidant genes were expressed in the FR and SR, whereas extracellular PODs were expressed in the FR and TR in a stage-specific manner. The antioxidant gene expression patterns should help elucidate the functions of these genes during root development. Antioxidant enzyme activities and expression in different root parts remain to be studied under abiotic and biotic stresses. Further study of antioxidant mechanisms at different root developmental stages under environmental stress conditions will be useful for developing cultivars and agricultural practices that increase sweetpotato yield.

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