

# Enhanced Reactive Oxygen Species Scavenging by Overproduction of Superoxide Dismutase and Catalase Delays Postharvest Physiological Deterioration of Cassava Storage Roots<sup>1[C][W][OA]</sup>

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Postharvest physiological deterioration (PPD) of cassava (*Manihot esculenta*) storage roots is the result of a rapid oxidative burst, which leads to discoloration of the vascular tissues due to the oxidation of phenolic compounds. In this study, coexpression of the reactive oxygen species (ROS)-scavenging enzymes copper/zinc superoxide dismutase (MeCu/ZnSOD) and catalase (MeCAT1) in transgenic cassava was used to explore the intrinsic relationship between ROS scavenging and PPD occurrence. Transgenic cassava plants integrated with the expression cassette p54::MeCu/ZnSOD-35S::MeCAT1 were confirmed by Southern-blot analysis. The expression of MeCu/ZnSOD and MeCAT1 was verified by quantitative reverse transcription-polymerase chain reaction and enzymatic activity analysis both in the leaves and storage roots. Under exposure to the ROS-generating reagent methyl viologen or to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), the transgenic plants showed higher enzymatic activities of SOD and CAT than the wild-type plants. Levels of malondialdehyde, chlorophyll degradation, lipid peroxidation, and H<sub>2</sub>O<sub>2</sub> accumulation were dramatically reduced in the transgenic lines compared with the wild type. After harvest, the storage roots of transgenic cassava lines show a delay in their PPD response of at least 10 d, accompanied by less mitochondrial oxidation and H<sub>2</sub>O<sub>2</sub> accumulation, compared with those of the wild type. We hypothesize that this is due to the combined ectopic expression of Cu/ZnSOD and CAT leading to an improved synergistic ROS-scavenging capacity of the roots. Our study not only sheds light on the mechanism of the PPD process but also develops an effective approach for delaying the occurrence of PPD in cassava.

Cassava (*Manihot esculenta*) produces an acceptable yield under the adverse climatic and nutrient-poor soil conditions occurring in some tropical and subtropical

regions; therefore, it is recognized as being an important food security crop (Sayre et al., 2011). Nevertheless, the rapid postharvest physiological deterioration (PPD) of its storage roots, a unique phenomenon compared with other root crops, renders the roots unpalatable and unmarketable within 24 to 72 h of harvest, thereby adversely impacting farmers, processors, and consumers alike (Rickard, 1985; Reilly et al., 2004, 2007). While the exclusion of oxygen by storing and transporting the roots in plastic sacks, or coating individual roots with paraffin wax, has been used to minimize the problem of PPD in cassava, these approaches only provide solutions for wealthier consumers or expatriate communities and are not generally economically viable for such a low-value commodity (Reilly et al., 2004).

Early studies identified phenolic compounds whose accumulation and oxidation led to the discoloration of vascular tissues; these included scopoletin, scopolin, esculin, and proanthocyanidins (Rickard, 1985). Various other secondary metabolites including volatile compounds, which are associated with the early stages of cassava root PPD, had been detected (Buschmann et al., 2000; Iyer et al., 2010). The application of cycloheximide to inhibit protein synthesis (Beeching et al., 1998) confirmed that PPD was an active process, rather than a

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degenerative one, involving changes in gene expression, protein synthesis, and phenolic compound synthesis (Huang et al., 2001). Reactive oxygen species (ROS) increased very early during PPD (Iyer et al., 2010). Cellular processes, including ROS turnover, programmed cell death, defense pathways, signaling pathways, and cell wall remodeling, have been shown to be active during the deterioration response using complementary DNA microarray and isobaric tags for relative and absolute quantification-based PPD proteome analyses (Reilly et al., 2007; Owiti et al., 2011). A burst of superoxide is detected within minutes of harvesting, followed by peaks of other ROS and increased activities of ROS-scavenging enzymes (Reilly et al., 2001, 2004, 2007), suggesting that ROS plays a major role in, and may in fact initiate, the PPD response. A recent study showed that PPD is cyanide dependent, presumably resulting from cyanide-dependent inhibition of respiration (Zidenga et al., 2012). Therefore, while there is considerable evidence linking ROS accumulation to PPD, there is a need to confirm the intrinsic relationship between ROS accumulation and PPD occurrence and their regulation.

ROS, which include the superoxide anion radical, hydroxyl radical, and hydrogen peroxide ( $H_2O_2$ ), are potentially harmful to the cell and cause oxidative damage to proteins, DNA, and lipids (Apel and Hirt, 2004). Plants have developed efficient nonenzymatic and enzymatic detoxification mechanisms to scavenge ROS. Nonenzymatic antioxidants include the major cellular redox buffers, ascorbate and glutathione, as well as tocopherol, flavonoids, alkaloids, and carotenoids. Enzymatic ROS-scavenging mechanisms in plants include superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), and glutathione peroxidase (EC 1.11.1.7; Asada, 1999; Apel and Hirt, 2004; Mittler et al., 2004). SOD, acting as the first line of defense against ROS, converts superoxide to  $H_2O_2$ . There are essentially three types of SODs containing manganese, iron, or copper plus zinc as prosthetic metals (Raychaudhuri and Deng, 2000). The resultant  $H_2O_2$  can be detoxified to oxygen and water by CAT or APX, which occur mainly in peroxisomes.

Several groups have addressed the overproduction of SOD (Gupta et al., 1993; Perl et al., 1993; McKersie et al., 1996, 2000) in the chloroplasts as a means to enhance tolerance to oxidative stress. In addition, transgenic plants expressing CAT had increased tolerance against various abiotic stresses (Shikanai et al., 1998; Miyagawa et al., 2000; Moriwaki et al., 2007). However, others have reported that the expression of either SOD or CAT alone led to no change in response to oxidative or environmental stress (Pitcher et al., 1991; Payton et al., 1997). These contradictory findings may be due to the complex network of plant antioxidant defenses and raise the possibility that a higher tolerance to oxidative stress might be achieved by pyramiding or stacking genes in a single genotype

(Halpin, 2005). Transgenic cotton (*Gossypium hirsutum*) expressing both glutathione reductase and APX improved the recovery of photosynthesis following exposures to 10°C and high photon flux density (Payton et al., 2001). Transgenic Chinese cabbage (*Brassica campestris* ssp. *pekinensis*) plants expressing both SOD and CAT in chloroplasts enhanced tolerance to sulfur dioxide and salt stress (Tseng et al., 2007). Coexpression of copper/zinc SOD (Cu/ZnSOD) and APX genes in the chloroplasts or cytosol of tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), and sweet potato (*Ipomoea batatas*) enhanced tolerance to multiple abiotic stresses, including the herbicide methyl viologen (MV), chilling, high temperature, and drought stress (Kwon et al., 2002; Tang et al., 2006; Lee et al., 2007; Faize et al., 2011).

As a clonally propagated, highly heterozygous crop plant, conventional breeding for tolerance to PPD via the cross hybridization between cassava and its relatives, although possible (Chavez et al., 2000), may not be straightforward and is time consuming. Some variation in PPD tolerance has been found in various cassava landraces (Contreras Rojas et al., 2009); for example, Morante et al. (2010) reported that cassava roots with high carotenoid levels were tolerant to PPD for up to 40 d after harvest. They attributed this character to the antioxidant properties of carotenoids, but the trait proved difficult to transfer to common cultivars. Pruning the plants a few days before harvest also delays PPD, but at the expense of a reduction in dry matter content of the root (van Oirschot et al., 2000). To date, traditional breeding alternatives do not appear to offer a practical solution, and PPD has remained a difficult problem to solve. Therefore, molecular and biochemical regulation of the PPD process using transgenic technology offers alternative approaches for controlling the PPD response.

Recently, transgenic cassava overexpressing the Arabidopsis (*Arabidopsis thaliana*) mitochondrial alternative oxidase (AOX 1A) gene were shown to delay PPD for up to 10 d by reducing ROS accumulation (Zidenga et al., 2012). The PPD response is an enzymatically mediated oxidative process in which ROS appear to play a dual role as both a signaling molecule that induces programmed cell death as part of a more general wound response and in oxidizing phenolic compounds to produce the visible symptoms of PPD, with wound repair and antioxidant defenses being too late or inadequate to contain these effects (Reilly et al., 2004). Both SOD and CAT activities increased significantly in storage roots after harvest, although too late to contain the PPD response (Reilly et al., 2001, Owiti et al., 2011); additionally, the antioxidant effects of the two enzymes are directly linked through their converting superoxide to  $H_2O_2$  and  $H_2O_2$  to oxygen and water, sequentially. In this study, we used transgenic cassava overexpressing cassava SOD and CAT to study their synergized effect on the intrinsic relationship between ROS scavenging and PPD occurrence, which also leads to an appropriate approach for

delaying the deterioration of cassava storage roots after harvest.

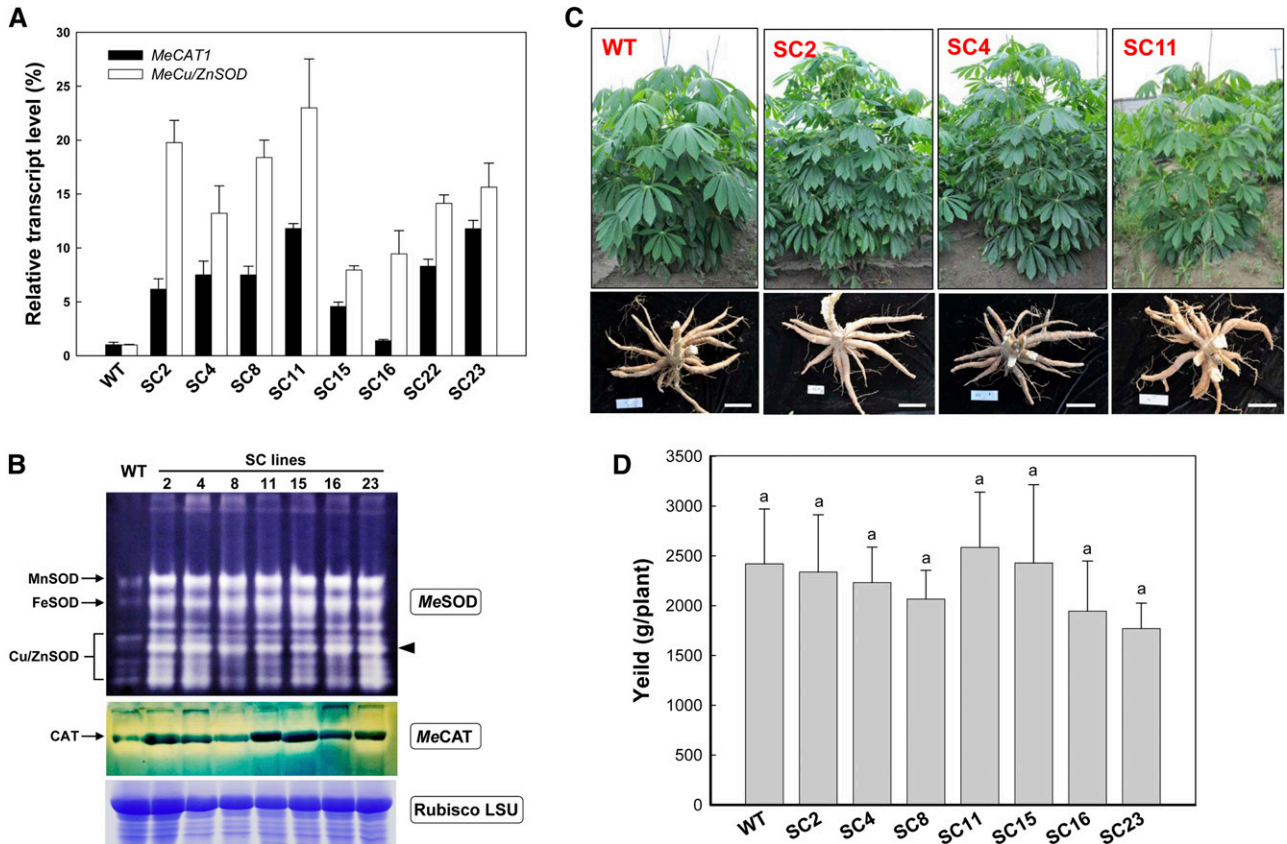
**RESULTS**

**Molecular Characterization of Transgenic Plants and Increased Expression of SOD and CAT1 Showed No Visible Phenotypic Changes**

In order to test how ROS regulates the PPD response, *MeCu/ZnSOD* and *MeCAT1* stacked in the binary vector pC-P54::MeCu/ZnSOD-35S::MeCAT1 (Supplemental Fig. S1A) were used to produce transgenic cassava. Since ROS were mainly generated from the cells of vascular tissues, the expression of the *MeCu/ZnSOD* gene were driven by the vascular-specific promoter p54/1.0 from cassava (Zhang et al., 2003), while the *MeCAT1* gene was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in order to avoid transgene silencing caused by using the same promoter.

Nine independent transgenic plant lines (named SC for short) were regenerated from transformed embryogenic suspension on hygromycin-containing medium. In vitro plants were tested by PCR for the presence of the *MeCu/ZnSOD* and *MeCAT1* transgenes (data not shown). The copy number of the transgenes in the transgenic plants was assessed by Southern-blot analysis using the *Xba*I-digested genomic DNA hybridized with digoxigenin-labeled *hygromycin phosphotransferase (HPT)* and *CAT1* probes (Supplemental Fig. S1B). The transgenic cassava plants were truly independent lines and have a pattern of transfer DNA integration ranging from one to two copies.

Different transcriptional levels of *MeCu/ZnSOD* and *MeCAT1* in the eight single-copy lines compared with the nontransformed control were detected by quantitative reverse transcription (qRT)-PCR (Fig. 1A). The expression of *MeCu/ZnSOD* and *MeCAT1* increased to 20- and 10-fold, respectively. The increased enzymatic



**Figure 1.** Analyses of cassava SOD and CAT transcript and abundance and phenotypic evaluation of transgenic plants harvested from the field. A, qRT-PCR analysis of *MeCu/ZnSOD* and *MeCAT1* expression levels both in wild-type (WT) and transgenic cassava. Total RNA was extracted from leaves, and the data are shown relative to the wild type, using  $\beta$ -actin as an internal control. Data are presented as means  $\pm$  SD of three independent RNA samples. B, SOD and CAT isoforms in leaves of wild-type and transgenic plants detected by activity staining of a nondenaturing polyacrylamide gel. Three SOD isoforms, MnSOD, FeSOD, and Cu/ZnSOD, are indicated. A substantial increase of the cytoplasmic Cu/ZnSOD is highlighted by the black arrowhead. The Rubisco LSU protein was used as a loading control. C and D, Normal growth of transgenic plants with fully developed storage roots (C) and unchanged yield (D) in comparison with the wild type in the field. Bars = 15 cm. No significant difference was found by Duncan's multiple comparison tests at  $P < 0.05$ . [See online article for color version of this figure.]

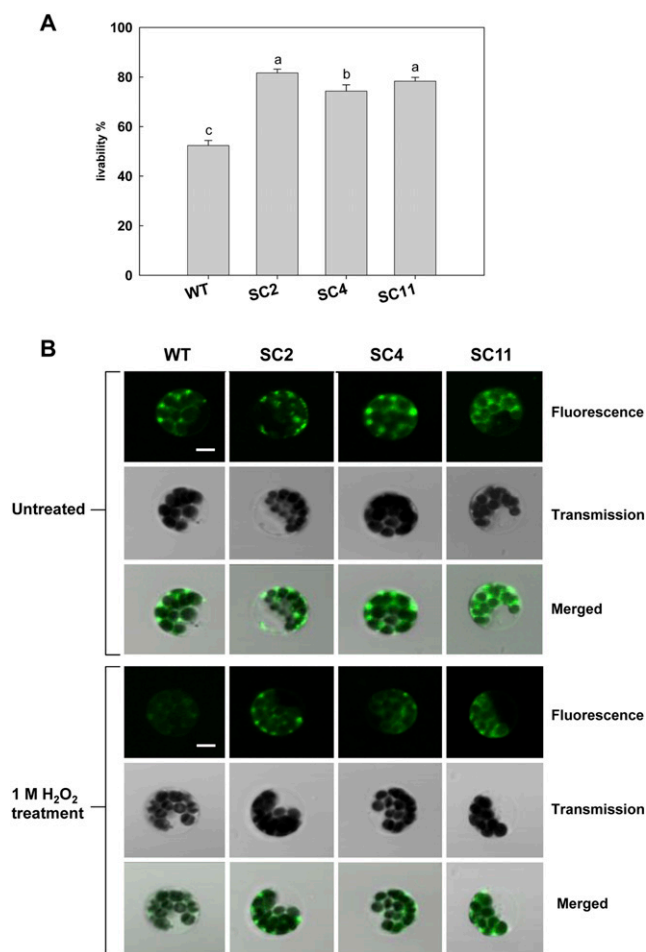
abundances of the different SOD and CAT isoforms were detected, and the results are shown in Figure 1B. A typical SOD isoenzyme banding pattern (Chen and Pan, 1996), which consisted of manganese SOD (MnSOD), iron SOD (FeSOD), and Cu/ZnSOD, was found. In all transgenic lines, MnSOD, FeSOD, and Cu/ZnSOD bands were stronger than those of the wild type. Comparison of the intensities of the Cu/ZnSOD bands showed that one band was greatly enhanced compared with the wild type (Fig. 1B, arrowhead), due to the overproduction of the cytoplasmic MeCu/ZnSOD protein in the transgenic lines. The transgenic lines also showed a stronger CAT isoenzyme band than the wild type. These results demonstrated the elevated levels of SOD and CAT enzymes in transgenic plants.

None of the transgenic plants, grown in either the greenhouse or the field, showed any visible phenotypic and yield differences compared with the wild type under the same growth conditions (Fig. 1, C and D), indicating that the overexpression of the stacked MeCu/ZnSOD and MeCAT1 genes occurs without phenotypic defects in the transgenic cassava.

### The Transgenic Plants Have Enhanced Tolerance to Oxidative Stresses

To assess ROS-scavenging capacity in the transgenic cassava, tolerance to exogenous  $H_2O_2$  stress using protoplasts of transgenic plants was determined. Under 1 M  $H_2O_2$  treatment, the viability of transgenic protoplasts from the leaves of SC2, SC4, and SC11 remained 80%, 75%, and 79%, respectively, which was significantly higher than that of the wild type, which only showed 50% viability (Fig. 2A). The protoplasts isolated from all cassava leaves showed intense green fluorescence under the untreated condition (Fig. 2B). When treated with 1 M  $H_2O_2$ , only a diffuse and weak fluorescent signal was observed, after the rhodamine 123 reaction, in the wild-type protoplasts, in contrast to the transgenic protoplasts, which still exhibited a strong green fluorescence (Fig. 2B). These results imply that the transgenic cells have enhanced oxidative stress tolerance compared with wild-type cells.

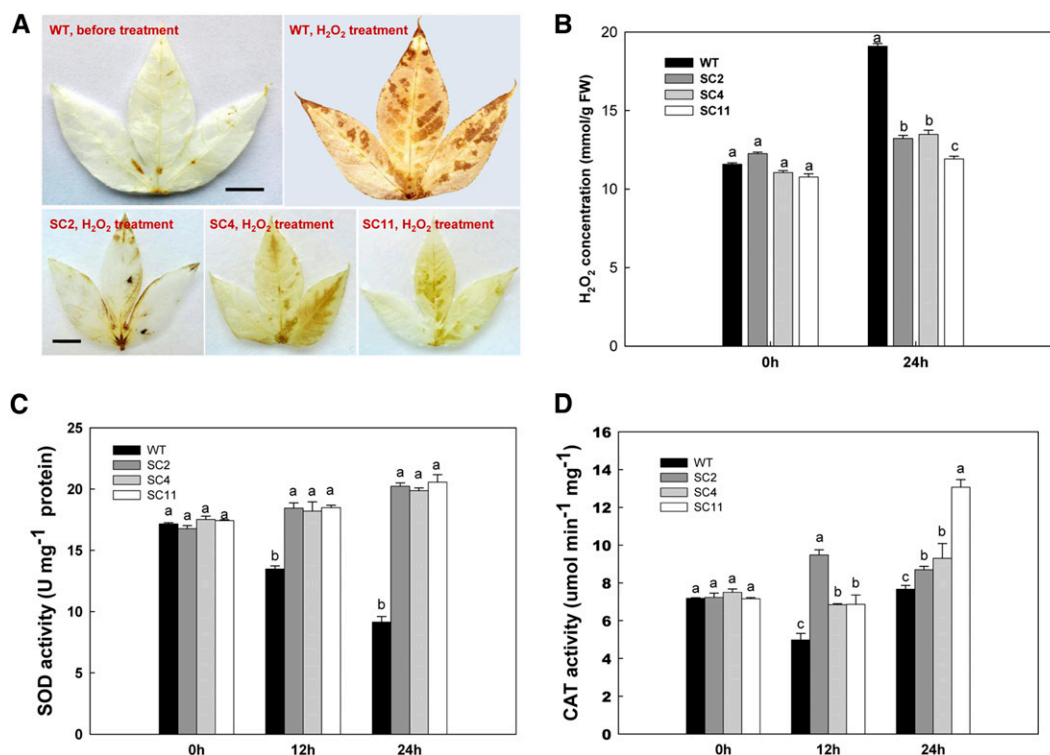
To confirm the enhanced ROS-scavenging capacity of the transgenic plants at the plant level, these were treated with 0.5 M  $H_2O_2$  oxidative stress. Before treatment, wild-type leaves showed only a basal level of  $H_2O_2$  production, as indicated both by 3,3'-diaminobenzidine (DAB) staining and colorimetric detection (Fig. 3, A and B). At this time point, no significant difference was found between wild-type and transgenic plants (Fig. 3B). After 0.5 mM  $H_2O_2$  treatment for 24 h, wild-type leaves accumulated  $H_2O_2$  significantly, mostly in epidermal cells, and reached a concentration of 19.1 mmol  $g^{-1}$ , a 1.65-fold change compared with untreated ones (Fig. 3B). In contrast to the wild type, transgenic leaves showed much less  $H_2O_2$  accumulation, as evidenced by slight



**Figure 2.** Changes in protoplast viability and mitochondrial membrane integrity in the presence of 1 M  $H_2O_2$ . A, The viability of cassava mesophyll protoplasts after  $H_2O_2$  treatment was estimated by fluorescein diacetate staining. Data are represented as means of six replicates  $\pm$  SD (more than 300 cells were counted for each experiment per genotype). Values labeled with different letters (a, b, and c) are significantly different by Duncan's multiple comparison tests at  $P < 0.05$ . B, Mitochondrial membrane integrity under 1 M  $H_2O_2$  stress. Cassava protoplasts were stained with rhodamine 123, and the fluorescent signal was observed with a confocal microscope. Bars = 5  $\mu$ m. [See online article for color version of this figure.]

DAB staining in leaves (Fig. 3A) and a low level of  $H_2O_2$  concentration (Fig. 3B). For example, the  $H_2O_2$  concentration in the leaves of SC2 was 13.2 mmol  $g^{-1}$ , only a 7.8% increase. The accumulation of  $H_2O_2$  in these transgenic lines was only about 70% of the wild type.

Without  $H_2O_2$  treatment, both wild-type and transgenic leaves showed only basal SOD and CAT activities, with no significant difference between them (Fig. 3, C and D). After treatment, the SOD activity of the wild type decreased over the time course. At 24 h, SOD activity was reduced to 52.5% of that at 0 h. However, a consistent increase of SOD activity was detected in the leaves of all three transgenic lines. For example, in SC2, the activity increased from 16.8 units  $mg^{-1}$  at 0 h to 20.2 units  $mg^{-1}$  protein by 24 h, about a 20% increase (Fig. 3C). CAT activity was also affected by  $H_2O_2$  treatment



**Figure 3.** Enhanced tolerance to H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress in transgenic leaves. A, H<sub>2</sub>O<sub>2</sub> accumulation in leaves detected by DAB staining. Bars = 0.5 cm. B, Changes in the levels of H<sub>2</sub>O<sub>2</sub> concentration between wild-type (WT) and transgenic cassava during 0.5 M H<sub>2</sub>O<sub>2</sub> treatment. C and D, Changes in SOD (C) and CAT (D) activities between wild-type and transgenic cassava during H<sub>2</sub>O<sub>2</sub> treatment. Values represent means of three independent experiments ± sd. Values labeled with different letters (a, b, and c) are significantly different by Duncan's multiple comparison tests at  $P < 0.05$ . [See online article for color version of this figure.]

(Fig. 3D). Unlike SOD, CAT decreased its activity at 12 h (about 32.2% compared with 0 h) but recovered by 24 h in the wild type. The CAT of transgenic leaves showed increased activity after H<sub>2</sub>O<sub>2</sub> treatment, especially in SC11: the activity level at 24 h increased 2-fold as compared with 0 h (Fig. 3D), reaching 13.1  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ . At 24 h, all transgenic leaves showed significantly higher activity in comparison with the wild type. These results confirm that the improved performance of transgenic cassava leaves against oxidative stress is due to elevated SOD and CAT activities.

The efficacy of endogenous ROS scavenging in transgenic leaves was tested using the ROS-generating reagent MV. When subjected to 100  $\mu\text{M}$  MV treatment for 2 d, cassava leaves were greatly affected, showing discoloration (Fig. 4A) and reduced chlorophyll content (Fig. 4B). The reduction of chlorophyll content in transgenic leaves was 38%, 33%, and 45% in SC2, SC4, and SC11, respectively, which was significantly less than the wild type (74%; Fig. 4B).

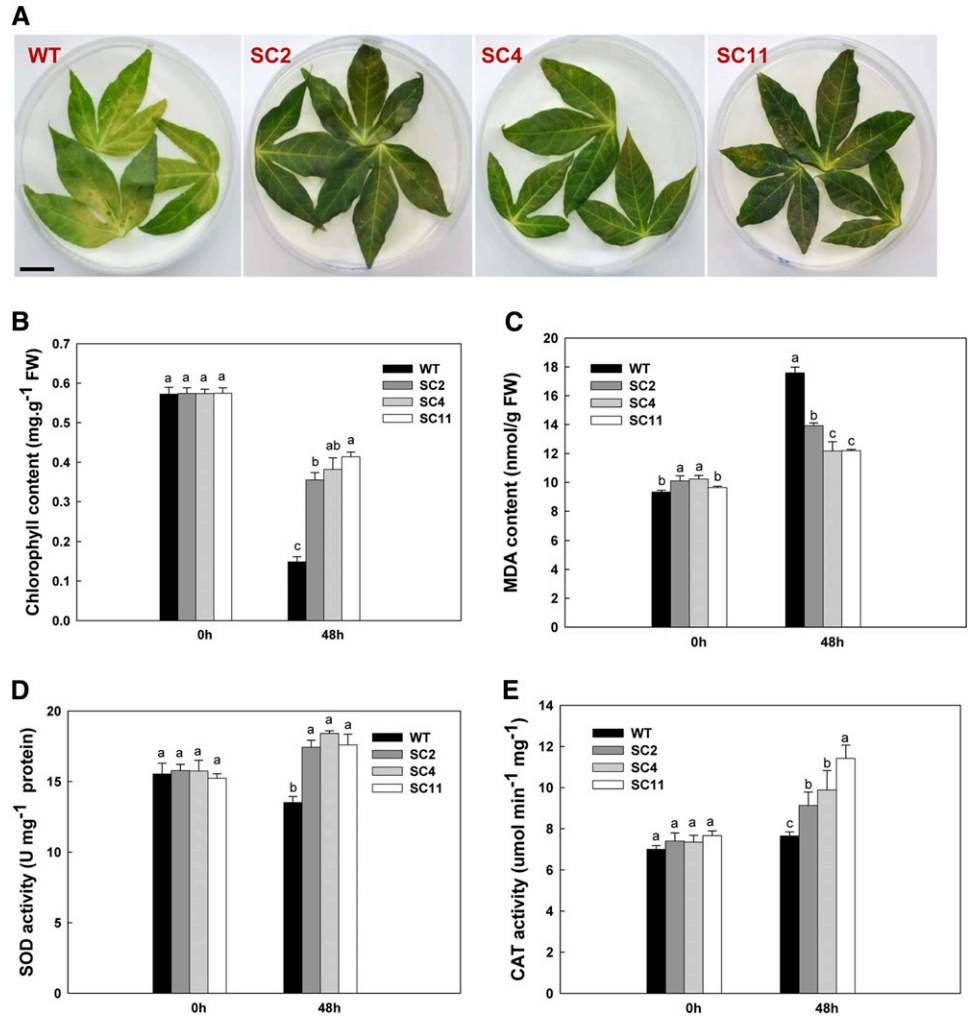
After the MV treatment, increased malondialdehyde (MDA) content, an indicator of lipid peroxidation, was observed in both wild-type and transgenic leaves (Fig. 4C). However, transgenic leaves only showed about a 13% increase in comparison with 50% in the wild type, indicating less cellular membrane damage in the

transgenic leaves (Fig. 4C). Additionally, both SOD and CAT activities were significantly enhanced (Fig. 4, D and E), with average increases of 30% for SOD and 20% for CAT in transgenic leaves, in contrast to their reduced activities in the wild type, results that were similar to those obtained with H<sub>2</sub>O<sub>2</sub> treatment. These data confirm that the coexpression of *MeCu/ZnSOD* and *MeCAT1* renders transgenic cassava leaves more resistant to both exogenous and endogenous oxidative stresses.

#### Storage Roots of Transgenic Cassava Show Delayed PPD Occurrence

Analysis of field-grown plants showed that the transgenic plants had no obvious difference in terms of root production and yield (Fig. 1, C and D). However, it was also pertinent to determine whether the enhanced capacity of ROS scavenging influenced the storage root PPD response. At 48 h post harvest, PPD symptoms could be seen in the storage roots of wild-type cassava; these roots showed the typical symptom of brown vascular streaking. More visible dark-brown coloration was observed in the starch-rich parenchyma section from 72 h, with 11% vascular discoloration level (Fig. 5A). Compared with the wild type, storage

**Figure 4.** Enhanced tolerance to MV-mediated oxidative stress in transgenic leaves. **A**, Leaves treated with 100  $\mu\text{M}$  MV showing the senescence phenotype of wild type (WT) plants and the stay-green phenotype of transgenic plants. Bar = 0.5 cm. **B** and **C**, Chlorophyll and MDA contents in the first leaf of MV-treated and untreated plants. FW, Fresh weight. **D** and **E**, Changes in SOD and CAT activities between wild-type and transgenic cassava during MV treatment. Data are presented as means  $\pm$  SD from triplicate independent measurements. Values labeled with different letters (a, b, and c) at the same time point are significantly different by Duncan's multiple comparison tests at  $P < 0.05$ . [See online article for color version of this figure.]

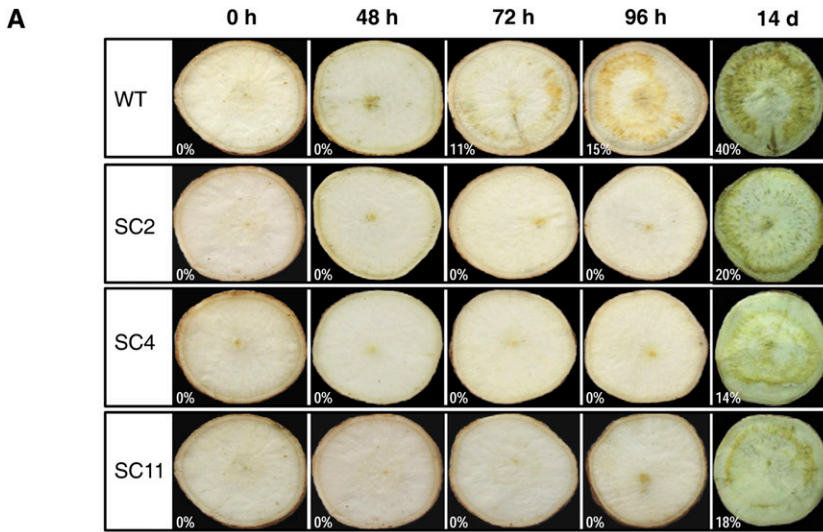


roots of the three transgenic lines did not show typical PPD symptoms even after 96 h (Fig. 5A), indicating a tolerance to PPD development. No visible PPD occurrence was noticed in 10 d. However, by 14 d after harvest, clear PPD symptoms could also be observed in their storage root sections (the vascular discoloration levels of the wild type, SC2, SC4, and SC11 were 40%, 20%, 14%, and 18%, respectively), although different lines responded differentially (Fig. 5A).

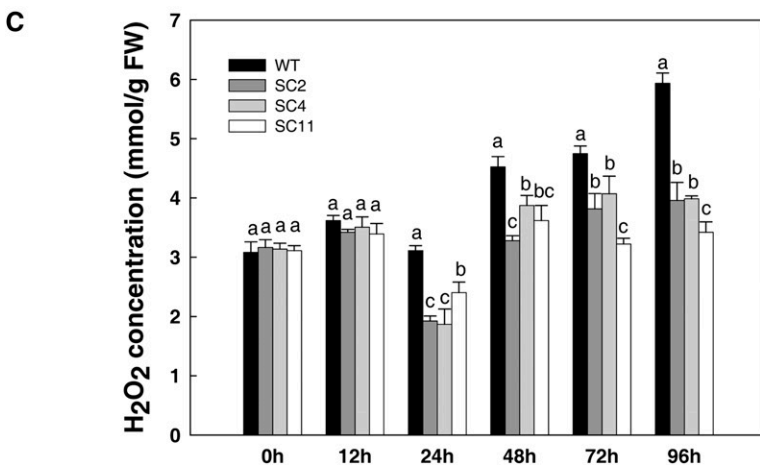
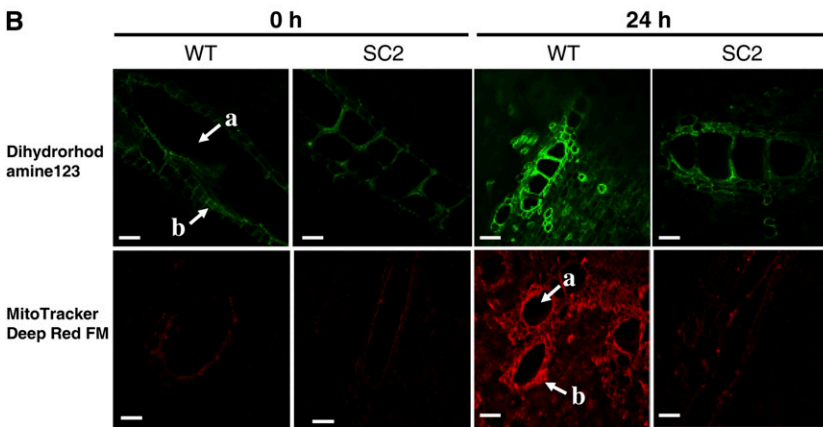
To test whether delayed PPD occurrence was related to a reduced oxidative burst, ROS generation and mitochondrial localization were assayed by the use of the oxidation-sensitive fluorescent probe dihydrorhodamine 123 (DHR) and MitoTracker-Deep Red FM, two mitochondrion-selective probes, on the storage roots of the wild type and SC2 (Fig. 5B). Immediately after harvest, the fresh storage roots from the wild type and SC2 showed only a very weak fluorescent signal along the xylem vessels (Fig. 5B), no difference being observed between the wild type and the transgenic line. After 24 h, bright fluorescence was detected, not only in the xylem vessels but also in the parenchyma cells of the wild-type storage roots, thereby showing an increased production and accumulation of ROS during the PPD

process (Fig. 5B); the fluorescent signal in the SC2 storage roots was much weaker, indicating either a relatively lower mitochondrial ROS generation or an improved capacity of ROS scavenging (Fig. 5B). Overall, ROS, especially H<sub>2</sub>O<sub>2</sub>, were mainly located in the cortical and internal storage parenchyma associated with xylem vessels at the onset of PPD.

To determine whether the additional overexpression of *MeCu/ZnSOD* and *MeCAT1* was involved in scavenging excess ROS in storage roots during the postharvest period, H<sub>2</sub>O<sub>2</sub> content at different PPD time points was measured. Generally, the trend of H<sub>2</sub>O<sub>2</sub> content in wild-type storage roots increased during the time course (Fig. 5C), although a small reduction at 24 h was observed, indicating a possible enhanced turnover of H<sub>2</sub>O<sub>2</sub> at that stage. After 12 h post harvest, all transgenic lines accumulated significantly less H<sub>2</sub>O<sub>2</sub> in their storage roots than the wild type (Fig. 5C). For example, the change of H<sub>2</sub>O<sub>2</sub> content in the SC2 sample at 12 and 96 h was from 3.42 to 3.96 mmol g<sup>-1</sup> fresh weight, only a 15.7% increase; for the wild type, a 64% increase of H<sub>2</sub>O<sub>2</sub> content was detected over the same period. Thus, there was a reduction in ROS accumulation in the transgenics compared with the wild type. We deduce that this is due



**Figure 5.** Delayed PPD occurrence of storage roots from transgenic cassava by visual and fluorescence determination. A, Visual detection of PPD occurrence using the International Center for Tropical Agriculture method (Wheatley et al., 1985; Morante et al., 2010). The levels of vascular discoloration are indicated by percentages using ImageJ processing software. B, Inhibition of mitochondrial ROS generation during the PPD process in transgenic storage roots detected by fluorescent probes. The fluorescence intensity of oxidized rhodamine was observed with a fluorescence microscope (Zeiss LSM 510 META) with excitation/emission of 488/515 nm and 635/680 nm for DHR and MitoTracker-Deep Red FM, respectively. a, Xylem vessel; b, bundle sheath. Bars = 20  $\mu$ m. C, Changes of H<sub>2</sub>O<sub>2</sub> concentrations during PPD between wild-type (WT) and transgenic cassava. Values labeled with different letters (a, b, and c) at the same time point are significantly different by Duncan's multiple comparison tests at  $P < 0.05$ . FW, Fresh weight. [See online article for color version of this figure.]



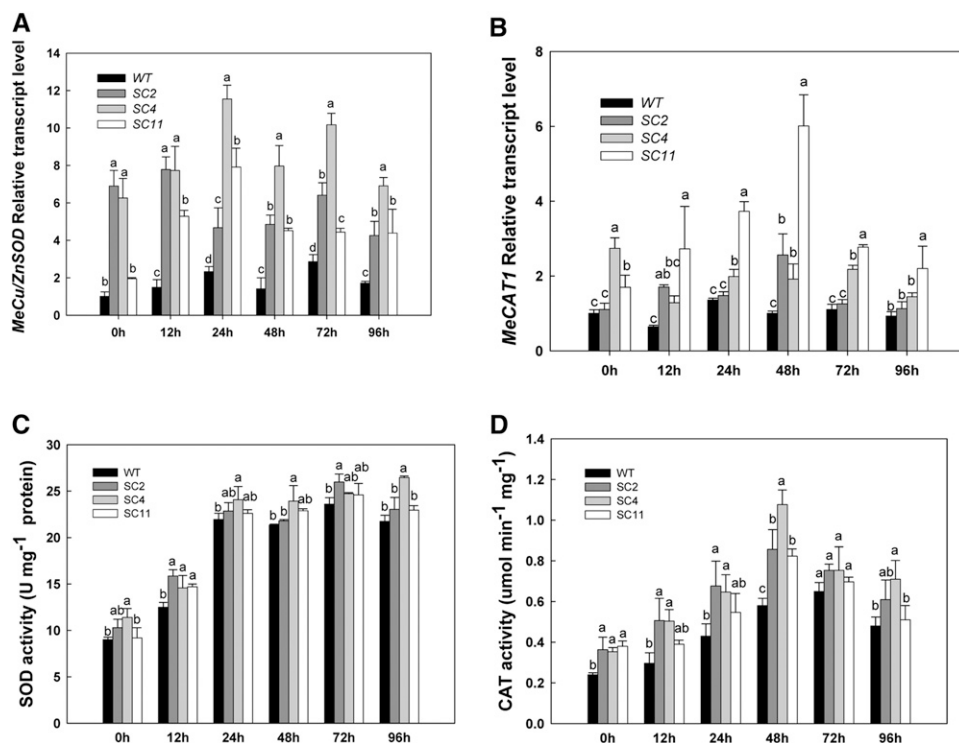
to increased ROS turnover, but it could also be due to reduced ROS production.

**Expression and Activity Patterns of SOD and CAT Enzymes during the PPD Process**

During the PPD process, relative higher levels of *MeCu/ZnSOD* and *MeCAT1* transcripts were detected

at all time points in transgenic samples than in the wild type by qRT-PCR analysis (Fig. 6, A and B). In the three transgenic lines, the expression levels of *MeCu/ZnSOD* were higher than those of *MeCAT1*, which indicated that the p54/1.0 promoter from cassava was stronger than the CaMV 35S promoter in the storage roots, consistent with the report by Zhang et al. (2003). Furthermore, the cassava promoter was not affected

**Figure 6.** Expression and activity patterns of SOD and CAT enzymes during the PPD process. A and B, Overall up-regulated expression of *MeCu/ZnSOD* and *MeCAT1* during PPD in transgenic plants compared with the wild type (WT). C and D, Higher levels of SOD and CAT activities during PPD in transgenic cassava compared with the wild type. Values labeled with different letters (a, b, and c) at the same time point are significantly different by Duncan's multiple comparison tests at  $P < 0.05$ .



or inducible by the PPD process (data not shown). The three independent lines also showed some variation in transcript patterns of *MeCu/ZnSOD* and *MeCAT1* between each line, although all showed significantly increased expression levels for both gene constructs compared with the wild type.

Total cellular SOD activities in wild-type and transgenic plants dramatically increased from 0 to 24 h during the postharvest period and remained at a high level from 24 to 96 h (Fig. 6, C and D). For the wild type, the SOD activity was 9 units  $\text{mg}^{-1}$  protein at 0 h and 23.6 units  $\text{mg}^{-1}$  protein at 72 h. For transgenic line SC2, its activity was 10.3 units  $\text{mg}^{-1}$  protein at 0 h and 26 units  $\text{mg}^{-1}$  protein at 72 h. The pattern of CAT activity was different from that of SOD, showing a gradual increase followed by a decline after 72 h for the wild type and after 48 h for transgenic lines (Fig. 6D). The SOD and CAT activities in storage roots after harvest reflect their central role in ROS turnover and scavenging, which is key to the modulation of the PPD response.

## DISCUSSION

Cassava PPD is a complex physiological and biochemical process involving changes of gene expression, protein synthesis, and the accumulation of secondary metabolites (Huang et al., 2001; Reilly et al., 2007). Studies on the mechanism of the PPD response are of importance, not only in increasing our molecular understanding of this unique biological phenomenon in an

important root crop but also for their potential in developing an effective approach to control postharvest losses. Several studies have shown that PPD is caused by an oxidative burst in the vascular bundle cells and starch-rich parenchyma cells of cassava storage roots and that ROS appear to play a central role both in triggering the onset of PPD and in symptom causation through the oxidation of phenolic compounds, as evidenced by the up-regulation of gene expression and protein biosynthesis related to ROS turnover (Reilly et al., 2007; Owiti et al., 2011). A recent study showed that cyanide released by mechanical damage of cassava roots caused during harvesting results in the buildup of ROS and that the overexpression of *Arabidopsis AtAOX* (a cyanide-resistant terminal oxidase in plants) in cassava reduced the accumulation of ROS and delayed PPD (Zidenga et al., 2012). However, while PPD was delayed, field-grown roots had reduced biomass compared with controls, which might be due to the heterologous expression of *AtAOX* affecting normal mitochondrial activity during root development (Zidenga et al., 2012).

In plants, ROS homeostasis, a delicate balance between cell defense and signaling, plays important roles in a diverse range of biological processes and responses to environmental stimuli (Alscher et al., 2002; Miller et al., 2010). In cassava, increases of ROS have been observed from immediately after root harvesting into the early stages of PPD, via ROS detection or by using radical-specific fluorescent imaging (Reilly et al., 2000, 2001, 2004; Iyer et al., 2010; Zidenga et al., 2012). In this study, we confirmed that the transcripts of



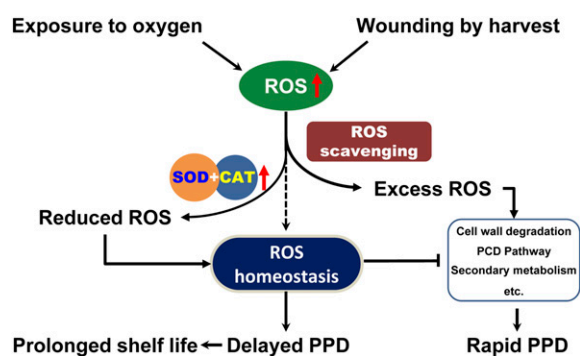
*MeCu/ZnSOD* and *MeCAT1* were up-regulated during PPD in the wild type (Fig. 6), which is consistent with previous reports (Reilly et al., 2007; Owiti et al., 2011). However, their profiles, together with the results of SOD and CAT enzymatic activity, were shown to be different, indicating different behaviors of the two genes in response to the oxidative burst in storage roots. We found that  $H_2O_2$  content increased slightly between 0 and 12 h and then suddenly dropped to a low amount by 24 h over the PPD time course. These data suggest that in the early stages, the superoxide radical is immediately converted to  $H_2O_2$  and that the available CAT was sufficient to catalyze the conversion of  $H_2O_2$  to water and oxygen, thereby leading to the reduction of  $H_2O_2$  concentration at 24 h (Fig. 5C). However, with the continued accumulation of more  $H_2O_2$ , the total capacity of CAT was not sufficient to scavenge the excess  $H_2O_2$ , despite its activity gradually increasing, a possible reason for the initiation of PPD symptoms, as highlighted by our model (Fig. 7). The additional expression of *MeCu/ZnSOD* and *MeCAT1* in the transgenic plants resulted in enhanced ROS scavenging, as observed through fluorescent detection (Fig. 5B), leading to the delay of PPD occurrence (Fig. 5A). This confirms the intrinsic relationship between ROS scavenging and PPD occurrence (Fig. 7).

Generally, more ROS will be produced in stressed cells, which leads to cellular damage (An et al., 2012). In transgenic cassava, leaves under MV and  $H_2O_2$  treatment showed stronger tolerance against these stresses and improved viability compared with the wild type (Figs. 2–4), demonstrating that the overexpression of *MeCu/ZnSOD* and *MeCAT1* reduced ROS accumulation in transgenic plants. The overexpression of SODs can lead to protection against specific stresses, implying that SOD may be the first line of defense against ROS (Gupta

et al., 1993; Perl et al., 1993; McKersie et al., 1996, 2000). But Tepperman and Dunsmuir (1990) were unable to detect high resistance to MV in tobacco plants that expressed a petunia (*Petunia hybrida*) chloroplast *Cu/ZnSOD* 50-fold compared with control plants. However, when the overexpression resulted in a moderate increase in SOD activity, the transgenic plants were tolerant to oxidative stress, as the equilibrium between oxygen radicals and  $H_2O_2$  was maintained (Perl et al., 1993). The authors suggested that elevating SOD without coelevating enzymes that remove  $H_2O_2$  could not provide protection against ROS toxicity. Importantly, CAT, a key  $H_2O_2$ -scavenging enzyme that is mainly located in peroxisomes, had been shown to be important in protecting plant cells against stresses; overexpression of CAT in tobacco or rice (*Oryza sativa*) improved paraquat, drought, or salt tolerance (Shikanai et al., 1998; Miyagawa et al., 2000; Moriwaki et al., 2007). CAT activity was higher in the less susceptible cultivars during the postharvest period (Reilly et al., 2001), while our data show that the ectopic overexpression of CAT led to delayed PPD, suggesting that high CAT activity may play a pivotal role in modulating PPD in cassava.

Several studies have demonstrated that gene stacking (e.g. of SOD and APX) could synergistically increase ROS turnover, thereby enhancing stress tolerance in a range of plant species (Kwon et al., 2002; Tang et al., 2006; Lee et al., 2007; Faize et al., 2011). In this study, the in vivo imaging of ROS using the fluorescent probe rhodamine-123, a stain readily sequestered by active mitochondria, in cassava mesophyll protoplasts or DAB staining of leaves showed the improved tolerance of transgenic cassava cells to oxidative stress caused by  $H_2O_2$  and MV (Figs. 3 and 4). MV treatment in the presence of light leads to the generation of superoxide radicals and  $H_2O_2$  in chloroplasts. The increased SOD and CAT enzyme activities were able to rapidly scavenge ROS at the site of generation as well as prevent the formation of hydroxyl radicals. Therefore, this study convincingly demonstrates that coexpression of the antioxidant enzymes SOD and CAT in transgenic cassava leads to a synergistic effect that not only reduces ROS levels but also delays cassava PPD (Fig. 7).

In plant cells, most of the ROS produced originate from chloroplasts or peroxisomes, but in nongreen tissues, or in the dark, mitochondrial ROS production predominates. Therefore, the fluorescent probe DHR and MitoTracker-Deep Red FM were used to monitor ROS in cassava storage root organelles during PPD. The DHR probe targets mitochondria and, when oxidized by  $H_2O_2$  or superoxide anion radical, yields fluorescent cationic and lipophilic probes, while MitoTracker-Deep Red FM just targets and labels mitochondria (Gomes et al., 2005; Swanson et al., 2011). Root parenchyma tissue stained with the two probes revealed a strong fluorescence (Fig. 5), which initially localized to the vicinity of the xylem vessels where vascular streaking symptoms occur. Thus, we showed that the early accumulation of  $H_2O_2$  as well as superoxide anion radicals in mitochondria in cassava roots leads to postharvest



**Figure 7.** Schematic illustration of the intrinsic relationship between ROS production, ROS scavenging, and ROS homeostasis for regulating PPD in cassava storage roots. Exposure to oxygen or mechanical wounding during harvest leads to increased ROS production in the storage root. Inefficient endogenous ROS scavenging results in excess ROS, which triggers rapid PPD responses, and stable ROS homeostasis in harvested cassava roots is never achieved. Ectopic expression of SOD and CAT leads to timely scavenging of excess ROS, thereby keeping the ROS homeostasis balanced and delaying PPD occurrence. [See online article for color version of this figure.]

deterioration. These data imply the centrality of ROS and their modulation in the PPD response, confirming the important role of ROS in the development of PPD (Reilly et al., 2007; Iyer et al., 2010; Zidenga et al., 2012).

In conclusion, coexpression of *MeCu/ZnSOD* and *MeCAT1* in cassava could dramatically improve ROS-scavenging ability, leading to reduced H<sub>2</sub>O<sub>2</sub> accumulation, improved abiotic stress resistance, and delayed PPD occurrence. It also confirms the current model of oxidative burst as a key player in initiating ROS and that enhanced ROS-scavenging capacity represses PPD occurrence.

## MATERIALS AND METHODS

### Plasmid Construction and Transformation

Cassava (*Manihot esculenta*) *Cu/ZnSOD* (GenBank accession no. AY642137) and *CAT1* (GenBank accession no. AY170272) were controlled by the vascular-specific promoter p54/1.0 (GenBank accession no. AY217353.1; Zhang et al., 2003) and the CaMV 35S promoter, respectively. The two expression cassettes were inserted into the binary vector pCambia1301 containing the hygromycin phosphotransferase gene (*hpt*) under the control of the CaMV 35S promoter to generate pC-P54::MeCu/ZnSOD-35S::MeCAT1 (Supplemental Fig. S1A). The construct was introduced into *Agrobacterium tumefaciens* strain LBA4404, which was then used for genetic transformation. Embryogenic callus induction of cassava TMS60444 and *A. tumefaciens*-mediated genetic transformation were performed as described by Zhang et al. (2000).

### Plant Materials

Four-week-old in vitro plantlets of transgenic and control plants were transplanted into pots (25 cm in diameter × 20 cm in height) containing 5 kg of well-mixed soil (soil:peat:perlite, 1:1:1) and grown in the greenhouse (16 h/8 h of light/dark, 30°C/22°C day/night). One-month-old plants were taken for subsequent physiological analysis. For field evaluation, 10 2-month-old plantlets per transgenic line and the wild type were planted on May 20, 2011, in the Wushe Plantation for Transgenic Crops, Shanghai (31°13'48.00"N, 121°28'12.00"E), and harvested on November 1, 2011. The entire trial plot was surrounded by a border row of nontransgenic plants to reduce edge effects. The performance of field plants was recorded regularly until harvest.

### Molecular Analysis of Transgenic Plants

Genomic DNA was isolated from in vitro-cultured cassava leaf tissue according to the procedure of Soni and Murray (1994). To screen positive transgenic plants, one primer was designed to bind the promoter region (5'-ATGGCCCTCCATTATTACACT-3' for p54/1.0 and 5'-TGTGAAGATAG-TGGAAAAGGAAGG-3' for CaMV 35S) and another one bound to the gene region (5'-AACAACTGCTGCCCTTCCTACAAT-3' for *MeCu/ZnSOD* and 5'-CTCAGGATGGTGTGATAAGAAGTC-3' for *MeCAT1*). Nontransformed plants were used as a control. PCR was performed using the following conditions: 95°C for 5 min, 30 cycles of 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. Finally, PCR products (759 bp for *MeCu/ZnSOD* and 823 bp for *MeCAT1*) were separated on a 1.0% agarose gel and visualized by ethidium bromide staining.

For Southern-blot analysis, 20 µg of genomic DNA was digested with *Xba*I, separated by electrophoresis on a 0.8% (w/v) agarose gel, and transferred onto a positively charged nylon membrane (Roche). *HPT* (1 kb) and *MeCAT1* (1.5 kb) were labeled with digoxigenin using the PCR DIG Probe Synthesis Kit (Roche). Hybridization and detection were performed according to the manufacturer's instructions using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche).

The expression levels of *MeCu/ZnSOD* and *MeCAT1* transgenes were determined by real-time qRT-PCR.  $\beta$ -Actin was used as a reference for normalization. Total RNA was extracted from cassava leaves and/or storage roots using the RNA Plant Plus Reagent (Tiangen). The RNA samples were digested with DNase I, and first-strand complementary DNA was synthesized

from 5 µg of total RNA from each sample using Moloney murine leukemia virus reverse transcriptase (Toyobo). qRT-PCR was performed using the Bio-Rad CFX96 thermocycler SYBR Green I Master mix (Toyobo) according to the supplier's protocols. PCR amplification was conducted by a 1-min pre-incubation step at 95°C followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 20 s. The primers were as follows:  $\beta$ -Actin (forward, 5'-TGAT-GAGTCTGGTCCATCCA-3'; reverse, 5'-CCTCCTACGACCCAATCTCA-3'), *MeCu/ZnSOD* (forward, 5'-ATGTTTCATGCCCTTGGAGAC-3'; reverse, 5'-GATCACCAGCATGACGAATG-3'), and *MeCAT1* (forward, 5'-TGGGAAA-CAACTTCCTGTC-3'; reverse, 5'-ACATCATCGAAGAACCAGGC-3').

### Isolation and Viability Assay of Mesophyll Protoplasts

In vitro-cultured cassava seedlings were grown at 25°C with light at 200 µmol m<sup>-2</sup> s<sup>-2</sup> and a photoperiod of 16 h/8 h of light/dark. The second and third fully expanded leaves (from the apex) were used as a source of protoplasts. Protoplasts were isolated as described by Anthony et al. (1995). Protoplast viability was determined by the uptake of fluorescein diacetate and observed using a fluorescence microscope (Nikon TE2000-S; Larkin, 1976). The purified protoplasts were treated with CPW9M solution (27.2 mg L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 101 mg L<sup>-1</sup> KNO<sub>3</sub>, 1,480 mg L<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 246 mg L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, and 9% mannitol, pH 7.0) supplemented with 1 M H<sub>2</sub>O<sub>2</sub> for 5 min and then stained with 0.01% fluorescein diacetate. Each sample was counted for about 300 cells. The viable percentage = (number of protoplasts with green fluorescence)/(number of total mesophyll protoplasts) × 100 (Duan et al., 2009).

### Analysis of Mitochondrial Integrity

Cassava mesophyll protoplasts were treated with 1 M H<sub>2</sub>O<sub>2</sub> for 5 min after staining with 10 µg mL<sup>-1</sup> rhodamine123 (Molecular Probes-Invitrogen). Fluorescence was detected using a confocal laser scanning microscope (Zeiss LSM 510 META) with excitation/emission of 488/515 nm. Twenty to 25 cells were measured for each sample.

### Treatment with MV and H<sub>2</sub>O<sub>2</sub>

Fully expanded healthy leaves were excised from plants and floated on different solutions of chemicals in 10-cm-diameter petri dishes. For the MV treatment, leaves were floated on 50 mL of 100 µM MV, while for the H<sub>2</sub>O<sub>2</sub> treatment, leaves were floated on 50 mL of 0.5 M H<sub>2</sub>O<sub>2</sub>. Leaves were incubated at 25°C for 48 h under continuous light.

### Determination of Chlorophyll Content

Chlorophyll was isolated from leaves according to the procedure of Hu et al. (2005). One gram of leaf tissues was ground and extracted with 10 mL of absolute ethyl alcohol. The absorbance of the supernatant was measured spectrophotometrically at 663, 646, and 470 nm, and chlorophyll content was calculated as described by Porra et al. (1989).

### Determination of Lipid Peroxidation

Lipid peroxidation in leaf tissues was measured in terms of MDA in the samples according to Dhindsa and Matowe (1981). Extraction was performed by homogenization of 1 g of leaf tissue with 10 mL of 10% (w/v) TCA, after which the homogenate was centrifuged at 10,000g for 10 min. Then, 2 mL of 10% TCA containing 0.67% (w/v) thiobarbituric acid was added to 2 mL of the supernatant. The mixture was incubated at 100°C for 15 min and centrifuged at 10,000g at 4°C for 10 min. The absorbance of the supernatant was recorded at 532 nm and corrected for nonspecific turbidity by subtracting the A<sub>600</sub>.

### Determination of H<sub>2</sub>O<sub>2</sub> Content and DAB Staining

The H<sub>2</sub>O<sub>2</sub> content was measured colorimetrically as described by Velikova et al. (2000). One gram of tissues was homogenized in an ice bath with 10 mL of 0.1% TCA. The homogenate was centrifuged at 10,000g for 15 min. One milliliter of the supernatant was added to 1 mL of 10 mM potassium phosphate buffer (pH 7.0) and 2 mL of 1 M potassium iodide. The absorbance of the supernatant was read at 390 nm. The content of H<sub>2</sub>O<sub>2</sub> was given on a standard curve.

H<sub>2</sub>O<sub>2</sub> was visualized by staining with DAB according to Thordal-Christensen et al. (1997). Detached leaves were infiltrated with 10 mL of DAB solution (1 mg mL<sup>-1</sup> DAB, pH 3.8) for 8 h. Leaves were immersed in 95% (w/v) boiling ethanol for 10 min to decolorize the chloroplast.

## Enzyme Assays

For the analysis of SOD isozymes, the protein extracts were separated on a 10% native polyacrylamide gel with a 4% stacking gel in standard Tris-Gly buffer (pH 8.3). Samples were electrophoresed at 100 V through the stacking gel for 20 min and at 120 V through the separating gel for 60 min. After electrophoresis, the gel was soaked in 0.1% (w/v) nitroblue tetrazolium (NBT) solution for 15 min, rinsed with distilled water, and transferred to 100 mM potassium phosphate buffer (pH 7.0) containing 0.028 mM riboflavin and 28 mM N,N,N',N'-tetramethyl-ethylenediamine for another 15 min. After being washed with distilled water, the gel was illuminated on a light box, with a light intensity of 30 mE m<sup>-2</sup> s<sup>-1</sup> for 15 min, to initiate the photochemical reaction (Chen and Pan, 1996).

SOD activity was determined based on the method of Beauchamp and Fridovich (1971), which measured inhibition of the photochemical reduction of NBT at 560 nm. Each 3-mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM Met, 75 mM NBT, 2 mM riboflavin, 0.1 mM EDTA, and 100 mL of enzyme extract. The reaction was carried out in test tubes at 25°C under a light intensity of 5,000 lux. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction of NBT by 50%.

For the analyses of CAT isozymes, the protein extracts were separated on a 7.5% native polyacrylamide gel with a 3.5% stacking gel in standard Tris-Gly buffer (pH 8.3). Samples were electrophoresed in the same conditions as for the SOD isozymes. After electrophoresis, the gel was soaked in 0.01% H<sub>2</sub>O<sub>2</sub> solution for 5 min, washed twice in water, and incubated for 5 min in 1% FeCl<sub>3</sub> and 1% K<sub>3</sub>[Fe(CN)<sub>6</sub>] (Zimmermann et al., 2006).

CAT activity was determined by following the consumption of H<sub>2</sub>O<sub>2</sub> at 240 nm for 4 min (Aebi, 1983). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H<sub>2</sub>O<sub>2</sub>, and 200 mL of enzyme extract in a final volume of 2 mL at 25°C.

## Visual PPD Evaluation

Based on the method of PPD evaluation from the International Center for Tropical Agriculture (Wheatley et al., 1985; Reilly et al., 2004; Morante et al., 2010; Zidenga et al., 2012), the proximal and distal ends of cassava storage roots were removed immediately after harvest. Proximal ends were exposed in the air, and distal ends of the root were covered with Parafilm. Roots were stored at 21°C to 28°C and 70% to 80% relative humidity. Evaluation of root sections was conducted at 0, 12, 24, 48, 72, and 96 h. Quantitation of vascular discoloration was done on captured images using ImageJ image-processing and analysis software (<http://rsb.info.nih.gov/ij/>).

## Fluorescence Measurement of PPD in Storage Root

The formation of ROS was determined by DHR and MitoTracker-Deep Red FM, which detect mitochondria oxidation-sensitive specific localization (Joo et al., 2001; Miller et al., 2009).

Fresh cassava storage roots from wild-type and transgenic lines were cut into smaller segments about 5 mm in length and width, then immediately soaked in sodium phosphate buffer (0.1 M), DHR (50 μM; Molecular Probes-Invitrogen) and MitoTracker-Deep Red FM (250 nM; Molecular Probes-Invitrogen) were added to each solution for 10 and 20 min, respectively. Fluorescence was detected using a confocal laser scanning microscope (Zeiss LSM 510 META) with excitation/emission of 488/515 nm and 635/680 nm for DHR and MitoTracker-Deep Red FM, respectively.

## Statistical Analyses

All data are represented as means ± SD from at least three independent experiments with three replicates. Statistical analysis was conducted using ANOVA, which was performed by using SPSS Statistics 17.0 to Duncan's multiple comparison tests. A value of  $P < 0.05$  was considered a statistically significant difference.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY642137, AY170272, and AY217353.1.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Schematic presentation of the transfer DNA region of pC-P54::MeCu/ZnSOD-35S::MeCAT1 and Southern-blot analysis of transgenic cassava lines.

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