Running Head: Molecular mechanisms for the control of cassava root shelf life

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Category: Biochemical Processes and Macromolecular Structures
**Title:** Extending cassava root shelf life via reduction of reactive oxygen species production.

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Abstract

One of the major constraints facing the large scale production of cassava roots is the rapid postharvest physiological deterioration (PPD) that occurs within 72 hours following harvest. One of the earliest recognized biochemical events during the initiation of PPD is a rapid burst of reactive oxygen species (ROS) accumulation. We have investigated the source of this oxidative burst to identify possible strategies to limit its extent and to extend cassava root shelf life. We provide evidence for a causal link between cyanogenesis and the onset of the oxidative burst that triggers PPD. By measuring ROS accumulation in transgenic low cyanogen plants with and without cyanide complementation, we show that PPD is cyanide dependent, presumably resulting from cyanide-dependent inhibition of respiration. To reduce cyanide-dependent ROS production in cassava root mitochondria, we generated transgenic plants expressing a codon-optimized Arabidopsis mitochondrial alternative oxidase (AOX 1A) gene. Unlike cytochrome C oxidase, AOX is cyanide insensitive. Transgenic plants overexpressing AOX exhibited over a 10-fold reduction in ROS accumulation compared to wild-type plants. The reduction in ROS accumulation was associated with delayed onset of PPD by 14-21 days after harvest of greenhouse-grown plants. The delay in PPD in transgenic plants was also observed under field conditions but with a root biomass yield loss in the highest AOX expressing lines. These data reveal a mechanism for postharvest physiological deterioration in cassava based on cyanide-induced oxidative stress, and PPD control strategies involving inhibition of ROS production or its sequestration.
Introduction:

Cassava (Manihot esculenta Crantz) is a woody shrub of the Euphorbiaceae family, grown mainly for its edible tuberous roots (Lokko et al., 2007). Worldwide, it is the sixth most important crop after wheat, rice, maize, potato and barley (Lebot, 2009). In the tropics, where it is a major staple food crop, cassava is the 4th most important source of calories (Bradbury, 1988). Tolerance to extreme environments such as drought and poor soils has made it an important food security crop in sub-Saharan Africa where it feeds more than 250 million people. In 2005, Africa produced an estimated 118 million metric tons of cassava (about 56% of total production) (Drapcho et al., 2008). One advantage of cassava is that it has a flexible harvesting date and can be left in the soil for up to two years, providing a ‘food bank’ for use as needed (Lebot, 2009).

In addition to its use as a staple food crop, cassava is also a potential biofuel crop owing to its high starch production. The Guangxi Zhuangzu autonomous region in southern China plans to expand cassava-based ethanol production from 139 million liters in 2007 to 1.27 billion liters in 2010 (Dai et al., 2005; Drapcho et al., 2008). Cassava is also an important source of industrial starch (for example Munyikwa et al., 1997; Ihemere et al., 2008). However, the short shelf-life of the roots (only two to three days) limits cassava’s economic and industrial potential. Harvested cassava roots undergo rapid postharvest physiological deterioration (PPD), which reduces their quality for market and consumption (Booth, 1976; Wenham, 1995; Buschman et al., 2000; Reilly et al., 2001; Westby, 2002; Reilly et al., 2004; Iyer et al., 2010). Cassava processing facilities, if any, must therefore be at or near the site of production to reduce postharvest losses. In most smallholder settings, this is not always a practical consideration. Thus, longer shelf life in farmer-preferred varieties would be desirable.

PPD is initiated by mechanical damage, which typically occurs during harvesting and progresses from the proximal site of damage to the distal end making the roots unpalatable within 72 hours (Wenham, 1995; Buschman et al., 2000; Iyer et al., 2010). This deterioration is an active process distinct from the secondary deterioration caused by microbial infection (Booth, 1976). A major visual symptom of PPD is vascular streaking.
resulting from occlusions in the vascular parenchyma by oxidized phenolics (Wenham, 1995). The rate of PPD is affected by environmental factors such as temperature, humidity and oxygen. Manipulation of these conditions can delay or hasten the process. For example, storage at 10°C and 80% humidity, waxing, and careful avoidance of physical damage can all delay PPD significantly (Wenham, 1995; Rickard, 1985; Plumbey and Rickard, 1991). While storage conditions and practices can go a long way in controlling PPD, the development of long shelf life varieties of cassava remains the most desirable strategy since no post-harvest intervention would be required.

Several studies have been carried out to understand the biochemistry of PPD (e.g. Buschman et al., 2000; Huang et al., 2001; Reilly et al., 2001; Reilly et al., 2004; Iyer et al., 2010). These studies have placed reactive oxygen species (ROS) production as one of the earliest events in the deterioration process. In plants, ROS are continuously produced as byproducts of aerobic respiration (Apel and Hirt, 2004). Under normal conditions, the plant has several mechanisms to scavenge ROS, preventing or ameliorating their toxicity. Under conditions of stress, however, the equilibrium between production and scavenging of ROS is disturbed, resulting in a rapid increase in the build-up of ROS known as an oxidative burst (Apostol et al., 1989).

In cassava roots, an oxidative burst occurs within 15 minutes of harvest (Reilly et al., 2004). Other early events include increased activity of enzymes that modulate ROS levels, such as catalase, peroxidase and superoxide dismutase (Reilly et al., 2001; Buschmann et al., 2000; Iyer et al., 2010). Further evidence in support of a role of oxidative stress in PPD comes from the observation that cassava cultivars that have high levels of β-carotene (which quenches ROS) are less susceptible to PPD (Sanchez et al., 2005). Earlier studies also reported a decline in phospholipid content during PPD, indicating membrane degradation, a known symptom of oxidative damage (Wenham, 1995).

While the role of oxidative stress in PPD has been established, the early events that trigger the oxidative burst have not been identified. In this study, we examine the role of
cyanogens in the oxidative burst observed during the onset of PPD. We show that cyanide released during mechanical damage in cassava roots results in the build-up of reactive oxygen species. Further, we show that overexpression of alternative oxidase (a cyanide-resistant terminal oxidase in plants) in cassava storage roots reduces accumulation of ROS and delays PPD by 10-21 days under greenhouse and field conditions.

**Results:**

**The oxidative burst in damaged cassava roots is cyanogen-induced**

Previous studies have shown that mechanical injury of cassava storage roots triggers cyanogenesis (McMahon et al. 1995) and an associated burst of acetone from cyanohydrins detected throughout the storage root (Iyer et al., 2010), followed by the production of ROS (Reilly et al. 2004). Cyanogenesis in cassava is induced during rupture of the vacuole, where linamarin is stored, followed by the production of cyanide in a two step process initially catalyzed by the cell wall localized enzyme linamarase and in leaves accelerated by hydroxynitrile lyase activity (Mkpong et al., 1990; White et al., 1998). To investigate whether there was a causal link between cyanogenesis and the oxidative burst, the production of ROS was measured in sectioned *in vitro* roots of wild-type cassava plants, and transgenic plants with reduced (< 1% of wild type) cyanogen levels, using two methods. In the first method, hydrogen peroxide accumulation was measured by incubating root sections in the presence of the hydrogen peroxide sensitive dye, 3, 3 diaminobenzidine. The intensity of the resulting brown coloration was quantified as a measure of hydrogen peroxide levels. Transgenic low cyanogen plants with less than 1% of wild-type linamarin levels (Cab1-1, Cab1-2 and Cab1-3; Siritunga and Sayre, 2003) had 2 to 8-fold lower dye-detectable hydrogen peroxide levels relative to wild-type plants (Figure 1A). In the second method, roots were exposed to the ROS-sensitive fluorescent dye 2′, 7′-dichlorofluorescein diacetate (H2DCFDA) and ROS-induced fluorescence was detected by laser confocal microscopy. ROS production, as indicated by elevated dye fluorescence, was as much as 11-fold lower in transgenic low...
cyanogen plants than in wild-type plants suggesting that cyanogen levels were correlated with ROS production (Figure 1B).

To ascertain whether the differences in ROS production were specifically due to differences in cyanogen content between wild-type and low cyanogen transgenic plants, we carried out biochemical complementation experiments in which the root slices of low cyanogen plants were pre-treated with 5 mM potassium cyanide (the cyanogenic potential of cassava roots typically ranges between 0.5-5 mM) before addition of H2DCFDA (McMahon et al., 1995). As indicated by the levels of H2DCFDA fluorescence, ROS production in low cyanogen plants increased upon pre-exposure to cyanide (Figure 2A). ROS accumulation increased two-fold in wild-type plants following addition of cyanide, while ROS accumulation increased between 2 and 16-fold in transgenic low cyanogen (Cab1) lines following addition of cyanide. Thus, exogenous cyanide complements the low oxidative burst phenotype in low cyanogen lines. These data show that the oxidative burst occurring in mechanically damaged cassava roots is induced by cyanogenesis, suggesting that a possible solution to cassava postharvest physiological deterioration is to reduce the cyanide-induced accumulation of reactive oxygen species.

The cyanide-induced oxidative burst in wounded cassava roots is mitochondrial

We hypothesized that cyanide release (Mkpong et al., 1990; McMahon et al., 1995; Siritunga and Sayre, 2003) was the primary event leading reactive oxygen species production via cyanide-induced inhibition of mitochondrial electron transfer and over-reduction of the upstream electron transfer complexes leading to ROS production (Moller, 2001). In root tissues there is an additional potential source of ROS production, the plasma membrane NADPH oxidase (Bhattacharjee, 2005). To determine if the plasma membrane NADPH oxidase could also account for the observed ROS production in detached roots, wild-type cassava roots were pre-treated with 100 µM diphenyl iodonium chloride (DPI), a non-specific inhibitor of the plasma membrane NADPH oxidase and screened for ROS production using the fluorescent dye, H2DCFDA. As indicated by
H2DCFDA fluorescence levels, pre-incubation of cassava root tissues with DPI reduced the accumulation of reactive oxygen species by only 20% (Figure 1B), suggesting that a substantive proportion of the observed oxidative burst was from sources other than plasma membrane NADPH oxidase. Collectively, these results indicate that cyanide inhibition of mitochondrial cytochrome oxidase is the major source ROS production in damaged cassava roots.

**Expression of Arabidopsis alternative oxidase prevents ROS accumulation in cassava roots**

Unfortunately, transgenic plants having low cyanogen levels due to inhibition of leaf-expressed CYP79D1/D2 do not grow well in the absence of ammonia due to the need to transport reduced nitrogen from leaves to roots via linamarin (Sayre et al., 2011). Therefore, an alternative strategy was needed to reduce cyanide-dependent ROS production or to sequester ROS produced following cyanogenesis. To reduce ROS production and presumably PPD in cassava roots, we overexpressed cyanide-insensitive mitochondrial alternative oxidase in cassava roots. Mitochondrial alternative oxidase provides a cyanide-insensitive pathway for reduction of oxygen facilitating the oxidation of over-reduced complex I and III that generate ROS following cyanide inhibition of cytochrome C oxidase (Maxwell et al., 1999). Cassava lines were genetically transformed via Agrobacterium-mediated transformation, using a codon-optimized Arabidopsis alternative oxidase (AOX1A) driven by the strong root-localized patatin promoter (see materials and methods). A total of 19 independent lines were generated and seven (PAOX1-7) were selected for further analysis. Expression of alternative oxidase in transgenic lines was confirmed by RT-PCR analysis (Figure 3B). Wild-type lines were negative for expression of the transgene.

To determine whether expression of AOX in transgenic cassava roots increased total alternative oxidase activity, mitochondria were isolated from tuberous roots and respiration rates were determined in the presence and absence of various mitochondrial electron transfer inhibitors. Alternative oxidase activity was defined as the rate of oxygen consumption resistant to cyanide and sensitive to SHAM. Figure 3C shows the results of
these analyses. The alternative oxidase activity for wild-type root mitochondria was 22.6 nmol O₂/mg protein/min, while for the transgenic lines it was between 37.5 nmol O₂/mg protein/min and 51.2 nmol O₂/mg protein/min (approximately a two-fold increase). The wild-type rates are within the range of previous cyanide-insensitive respiratory rates for cassava tuberous roots published by Passam (1976) ranging from 15-57 nmol of O₂ per mg protein per min. Significantly, wild-type cassava has substantially lower AOX rates activity compared to other crops such as soybean (66 nmol/mg protein/min; Kearns et al. 1992) and tobacco under nutrient stress (60 nmol/mg protein/min; Parsons et al. 1999). This may be due to the fact that the cassava storage root is metabolically less active compared to other plant roots.

Several AOX transgenic lines were also analyzed for ROS accumulation in root sections using the fluorescent dye H2DCFDA (Figure 4B). In the transgenic lines, ROS accumulation was reduced to barely detectable levels in line PAOX1-4 and was reduced by 4 to 14 times in line PAOX5-7. In addition, the hydrogen peroxide-specific stain DAB (Figure 4A) gave brown occlusions in root slices of wild-type lines (WT) but not in transgenic AOX lines. Thus, the overexpression of alternative oxidase substantially reduced the accumulation of reactive oxygen species in root slices.

**Expression of Arabidopsis alternative oxidase delays post-harvest physiological deterioration in cassava tubers**

The transgenic plants overexpressing alternative oxidase (AOX plants) were grown in the greenhouse for 4-6 months, during which time they developed small storage roots. The storage roots were assayed for PPD (see experimental procedures). Transgenic plants expressing alternative oxidase showed delayed PPD by at least 14 days (Figure 5A). There were no signs of vascular discoloration in transgenic AOX lines after 14 days while the wild-type exhibited vascular streaking. Transgenic AOX lines had no signs of PPD beyond two weeks after harvest, with PPD beginning to show only after 21 days in some lines. At 21 days post-harvest, PPD was mixed with secondary rotting (Figure 5B), but transgenic lines still showed minimal deterioration; less than 40% of the wild-type.
Three of the transgenic lines (PAOX2-4) were also grown under field conditions for one year in Puerto Rico. At both 5 and 10 days after harvest, roots of the transgenic lines had reduced signs of deterioration compared to wild-type. In particular, PAOX1 had 23% of the wild-type vascular discoloration level at day 5 and 46% at day 10 (Figure 6B). Delayed PPD (less than 50% of wild-type) was also observed in these plants at 10 days after harvest (Figure 6A and 6B). These results show that reducing reactive oxygen species accumulation by increasing alternative oxidase activity delays postharvest physiological deterioration in cassava.

**Overexpression of AOX can impact biomass yield of cassava tuberous roots**

Agronomic parameters were measured to determine whether AOX overexpression in cassava roots had any effect on the yield of cassava plants. Stem length in plants grown in greenhouse pots for 4 months was measured. Stem length in cassava is an important agronomic parameter since, along with the number of internodes, it determines the number of propagules that can be obtained from the plant. There was no significant difference between wild-type plants and transgenic lines in stem length except in lines PAOX5 (0.76±0.06) and PAOX6 (0.67±0.08), which were 15 and 17 percent lower than the wild-type (Table I). Under greenhouse growth conditions, all transgenic lines except PAOX1 had as much as a 3-fold increase in tuber fresh weight. This yield advantage was not, however, replicated under field conditions (Figure 6B) where only PAOX2 had higher yield (40% increase) compared to wild type, while PAOX1 and PAOX4 had substantially lower root fresh weight (93% and 82% lower respectively) than wild-type roots. Under field conditions root yield and shelf life were not directly related to AOX activity levels in greenhouse trials (Figure 3C).

**Discussion:**

Postharvest physiological deterioration is a major problem for cassava production. Estimates of economic losses due to PPD range from 5-25% of total potential crop income in Africa (Wenham, 1995). A recent *ex ante* estimate indicates that extending the
shelf life of cassava to several weeks would reduce financial losses by $2.9 billion in Nigeria alone over a 20-year period (Rudi et al., 2010). Several control strategies have been employed to reduce PPD in cassava. Farmers can harvest piecemeal, thereby minimizing storage constraints. However, keeping the crop in the soil for too long can affect quality and flavor as roots become woody (Westby, 2002). Additionally, in a semi-commercial setting, the land may need to be released for other uses. A more effective control strategy is oxygen exclusion, such as waxing the roots. This is generally not practical in smallholder settings, however, due to its high costs. A convenient control strategy for all farmers would be cultivars that have a longer shelf-life. Sanchez et al. (2005) showed that cassava cultivars with yellow roots (higher β carotene content) have a delayed onset of PPD by 1 to 2 days. In addition, Morante et al. (2010) surveyed different sources of germplasm and found delayed PPD (by up to 40 days) in three genotypes having high total carotenoid content (10.2-11.5 µg/g fresh weight compared to less than 1 µg/fresh weight in most of the other lines). However, cassava farming is also characterized by strong farmer preferences, such that yellow (high β carotene content) cultivars may not meet farmer preferences. Genetic transformation offers the possibility of transferring the trait to potentially any farmer-preferred variety (Sayre et al., 2011).

Postharvest physiological deterioration in cassava has been shown to be associated with an oxidative burst (Reilly et al., 2004). Recently, Iyer et al. (2010) showed SOD, catalase, and peroxidase were more highly expressed in regions closer to the site of mechanical damage. We show that this oxidative burst is due to cyanide production which is rapidly induced when cassava is mechanically damaged. Cassava produces potentially toxic levels of cyanogenic glycosides which break down to release cyanide following cellular disruption and release of the cyanogens from the vacuole (Miller and Conn, 1980; McMahon et al. 1995; Siritunga and Sayre, 2003; Siritunga et al. 2004). During cyanogenesis, glucose is cleaved from linamarin by the enzyme linamarase. The resulting acetone cyanohydrin is unstable and degrades spontaneously at pHs > 5.0 or temperatures > 35°C, or enzymatically by hydroxynitrile lyase (HNL) in leaves, releasing hydrogen cyanide and acetone (Cutler and Conn, 1981; White et al., 1994; Hughes et al., 1994; White and Sayre, 1995; White et al., 1998). However, cyanide release
generally does not happen in intact cells because linamarin is localized in the vacuole while the deglycosylase, linamarase, is localized in the cell wall and in laticifers (Mkpong et al., 1990; Hughes et al., 1994; McMahon et al. 1995). Harvesting operations generally cause tissue disruption, which allows linamarin and linamarase to come into contact, initiating cyanogenesis. Cyanide is a potent cellular toxin which inhibits the mitochondrial electron transport chain by tightly binding to the heme iron within cytochrome c oxidase, potentially leading to the accumulation of reactive oxygen species (Yip and Yang, 1988; Boveris and Cadenas, 1982). The availability of transgenic low cyanogen plants (Siritunga and Sayre, 2003), allowed us to investigate whether there was a causal link between cyanogenesis and the oxidative burst associated with PPD. Our results show that the oxidative burst is initiated by cyanide release occurring with mechanical wounding. Reduced accumulation of ROS in low cyanogen plants was complemented by addition of potassium cyanide in concentrations closely matching the cyanogenic potential of cassava. Inhibition of electron transport has been shown to result in increased ROS formation (Boveris and Cadenas, 1982). Thus, the cyanide released during cyanogenesis can cause accumulation of reactive oxygen species via inhibition of cytochrome oxidase in the respiratory electron transport chain. The $K_i$ for cyanide inhibition of cytochrome c oxidase is 10-20 µM, well below the cyanogenic potential of roots (1-5 mM) (Yip and Yang, 1988; Hell and Wirtz, 2008).

In roots, ROS can be produced from various sources including the plasma membrane NADPH oxidase and mitochondria (Moller, 2001; Sagi and Flurh, 2001). Experiments with diphenyl iodonium chloride, an inhibitor of the plasma membrane NADPH oxidase showed no substantial reduction in ROS production in its presence. This, together with the biochemical complementation experiments with potassium cyanide, support the hypothesis that ROS accumulation in damaged cassava roots is due to cyanide inhibition of mitochondrial electron transport chain (ETC). Mitochondria produce ROS at complexes I and III of the ETC (Bhattacharjee, 2011). It is estimated that up to 5% of oxygen consumed by isolated mitochondria results in the formation of ROS (Millar and Leaver 2000). Unlike animal mitochondria, the reduction of oxygen in plant mitochondria can occur by two different mechanisms (Vanlerberghe and McIntosh, 1997;
In addition to cytochrome C oxidase, plants possess an alternative oxidase (AOX), which catalyzes the tetravalent reduction of oxygen to water and branches from the main respiratory chain at ubiquinone (Vanlerberghe and McIntosh, 1997; Maxwell et al., 1999; Apel and Hirt, 2004). Importantly, the alternative oxidase pathway is cyanide-resistant, can function when the cytochrome pathway is impaired, and has been shown to play a role in lowering ROS formation in plant mitochondria by helping to modulate the redox state of upstream electron-transport components (Purvis, 1997; Popov et al., 1997; Maxwell et al., 1999; Finnegan et al., 2004). Alternative oxidase also has a considerably lower affinity for oxygen (km > 1 µM) compared to cytochrome C oxidase (km < 1 µM) (Medenstev et al., 2001). We hypothesized that overexpression of AOX could reduce accumulation of cyanide-induced ROS. Alternative oxidase provides an alternative route for electrons passing through the respiratory electron transport chain, which is not associated with a proton motive force, and thus reduces ATP generation. It is encoded by a small family of nuclear genes; AOX1, known for increased expression in response to stress, and AOX2 which is found in dicots and expressed in a constitutive and developmentally-regulated way (Juszczuk and Rychter, 2003). We expressed Arabidopsis AOX1A in cassava roots to reduce cyanide-induced ROS accumulation. As much as a four-fold reduction in ROS accumulation had previously been demonstrated in transgenic cultured tobacco cells overexpressing AOX (Maxwell et al., 1999). In addition, antisense suppression of AOX expression in tobacco (Nicotiana tabacum) resulted in cells with 2.5-fold higher levels of ROS compared to wild-type cells (Maxwell et al., 1999). We demonstrate that AOX overexpression in roots driven by the patatin promoter resulted in as much as an 18-fold reduction in ROS accumulation in cassava root slices.

Since postharvest physiological deterioration has been shown to be triggered by ROS accumulation, reducing accumulation of ROS in mechanically damaged cassava roots was hypothesized to delay postharvest physiological deterioration. Evaluation of root discoloration in transgenic plants expressing alternative oxidase showed a delay in the onset of PPD by at least two weeks. This window gives cassava producers enough time for transport and processing operations required after harvesting the crop. This strategy is
therefore anticipated to improve the transition of cassava production from subsistence to commercial. One transgenic line (PAOX2) did not show any signs of PPD 4 weeks after harvest under greenhouse conditions, suggesting potential for extended storage. These results suggest a model of PPD in cassava based on reactive oxygen species production (Figure 7). In this model, cyanide released on tissue damage causes an oxidative burst, which triggers postharvest physiological deterioration. Expression of AOX reduces ROS accumulation, resulting in delayed PPD. It is feasible, from this model, that PPD may also be controlled by increased activities (via transgenic expression) of enzymes or metabolites that scavenge reactive oxygen species, such as catalase, superoxide dismutase, peroxidase and carotenoids. The oxidative stress model for PPD has also been supported by the discovery of high beta carotene varieties of cassava that have a longer shelf life than low beta-carotene lines (Sanchez et al. 2006; Morante et al. 2010). Beta carotene is known to have antioxidant properties and is able to quench reactive oxygen species (Smirnoff, 2005).

Transgenic expression of alternative oxidase raises concerns, however, about possible effects on yield because the alternative pathway is associated with a reduced transmembrane potential and potential reduction in ATP synthesis. In addition, delayed PPD as a trait in conventional breeding in cassava plants has previously been associated with reduced dry matter content (Wenham, 1995). Our results show up to 3-fold increase in yield of cassava storage roots under normal greenhouse conditions in transgenic AOX lines, but up to 93% reduction in root yield in two of the three lines tested under more variable and presumably stressful field conditions. The yield changes in transgenic lines may be related to the proposed role of AOX in uncoupling carbon metabolism from ATP generation (Sieger et al. 2005; Vanlerberghe et al. 2009; Smith et al. 2009). Hansen et al. (2002) have shown that the plant growth rate is proportional to the rate of respiration and the efficiency by which it is coupled to phosphorylation. Control of AOX expression could provide a means by which respiration is adjusted to the energetic needs of cellular activities (Vanlerberghe et al. 2009). By modulating energy yield (ATP production and NADH consumption), AOX may also play a role in optimizing the rate of highly energy-consuming processes (Vanlerberghe et al. 2009). Since overexpression of AOX
has been linked to stress tolerance, it will be necessary to investigate the changes in yield under different stress conditions. For example, Smith et al. (2009) found 30-40% improved growth rates in Arabidopsis plants overexpressing AOX under salinity stress. Fiorani et al. (2005) found that altered levels of AOX protein result in leaf growth phenotypes in Arabidopsis plants grown under low temperatures. Under these conditions, antisense lines lacking AOX showed reduced leaf area, whereas lines over-expressing AOX1a showed increased leaf area compared to wild-type plants (Fiorani et al. 2005). These differences in leaf area between antisense lines, overexpressing lines and wild-type lines disappeared at normal growth temperatures (Fiorani et al. 2005). When transgenic tobacco suspension cells expressing an antisense construct of Aox1a were grown under nutrient deficiency, they accumulated significantly more biomass (over 2-fold in both low P and low N) than wild-type suspension cells (Sieger et al., 2005). Wild-type suspension cells are known to overexpress AOX under nutrient deprivation.Sieger et al (2005) suggested that AOX induction in wild-type cells suspension provided a means to reduce growth by uncoupling carbon metabolism from ATP generation and growth. The antisense lines without AOX had no such uncoupling and therefore unabated growth (Sieger et al., 2005). Overall, the cassava transgenic AOX plants generated here may improve commercialization of cassava by reducing postharvest losses and improve cassava yields by reducing losses due to PPD.

Materials and Methods:
Tissue culture propagation of plant material
Cassava plants were propagated in vitro in Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with Gamborg vitamins (Gamborg et al., 1968) and 2% (w/v) sucrose. In vitro plants were propagated in growth chambers at 28°C with 16 hours of light and 8 hours of darkness. Two wild-type lines were used (MCol 2215 and TMS 60444) as well as three previously generated low cyanide transgenic lines, Cab1-1, Cab1-2 and Cab1-3 in cultivar MCol 2215 (Siritunga and Sayre, 2003). In vitro cultures were maintained by subculturing nodal stems every four to eight weeks depending on the experiment.
Detection of Reactive Oxygen Species

Accumulation of reactive oxygen species (ROS) was measured using the method described by Maxwell et al. (1999). The method utilizes 2’, 7’-dichlorofluorescein diacetate (H₂DCF-DA; Molecular Probes), which emits green fluorescence on reaction with reactive oxygen species. Stock solutions (15 µM) of H₂DCF-DA were made in dimethyl sulfoxide (DMSO) and used immediately for the assays. Sections (1-2 mm) of in vitro cassava roots were incubated in H₂DCF-DA for 30 minutes, after which they were washed with distilled water and immediately analyzed on a Zeiss LSM 510 laser confocal microscope (Carl Zeiss Inc., North America) with excitation and emission wavelengths of 488 nm and 520 nm, respectively. Fluorescence intensity was quantified using ImageJ image processing software (NIH; http://rsbweb.nih.gov/ij/).

Detection of hydrogen peroxide in cassava roots

Hydrogen peroxide production was measured by an endogenous peroxidase staining procedure described by Rea et al. (2004). This procedure uses 3, 3-diaminobenzidine (DAB) which forms a reddish-brown precipitate on exposure to hydrogen peroxide. The DAB solution was prepared by dissolving 10 mg pellets of DAB in water to a concentration of 1.0 mg/mL. The pH of the solution was adjusted to 3.8. Sectioned cassava in vitro roots were incubated in 500 µL of 1.0 mg/mL DAB in closed microfuge tubes for 6-18 hours under light after which they were analyzed for hydrogen peroxide production. Images were captured under the Olympus DP20 light microscope (Olympus, PA). Quantitation of the intensity of coloration was done on captured images using ImageJ image processing software (NIH; http://rsbweb.nih.gov/ij/).

Inhibition of NADPH oxidase and mitochondrial electron transport chain

Experiments to inhibit NADPH oxidase in cassava roots were conducted using diphenyl iodonium chloride (DPI) as described by Orozco-Cárdenas et al. (2001). For this treatment, sectioned cassava roots from in vitro plants were pre-treated with 100 µM DPI in water for 30 minutes before analysis of reactive oxygen species by H₂DCF-DA fluorescence. Controls were treated with water for the same period. In experiments to complement cyanide in low cyanide transgenic cassava lines, Cab1-1, Cab1-2 and Cab1-
3 transgenic lines were pre-treated with 5.0 mM potassium cyanide (dissolved in water) for ten minutes prior to H$_2$DCF-DA treatment. Control plants were pre-treated with water for the same period.

**Design of alternative oxidase construct for cassava transformation**

A cyanide-insensitive mitochondrial alternative oxidase of *Arabidopsis thaliana* (*AtAOX1A*; At3g22370, GenBank Accession #: M96417) was codon-optimized for expression in cassava by a PCR-based method (Sremmer et al., 1995). The entire sequence including the 918 bp of *AtAOX1A* coding sequence and cloning sites was divided into 23 fragments with 42 bases each except the last one (19 bases). Twenty two forward primers were designed based on the first 22 fragments with codons optimized for cassava. Twenty reverse primers were designed so that they were overlapped with a half of two consecutive forward primers. A primary PCR was done with the mixture of the forward and reverse primers at the same molar ratio. The product was diluted prior to use in a secondary PCR. The secondary PCR was done with the diluted product of the primary PCR as a template and a primer set of **AOF1** (5’-CGCACCCGGGATATGGACACTAGACCCAACCATTGGAGGT-3’) and **AOR1** (5’-TGCCGAGCTCAATGATACCCAATTGGAGCTGGAGC-3’). **AOF1** primer contains SmaI site for cloning and the start codon, ATG, and **AOR1** has SstI site and the stop codon as indicated with the underlined bases. The final product was cloned into pUC19 using SmaI and SstI sites. Sequencing of the insert revealed a single base mutation, and the mutation was fixed using QuikChange® Site-Directed Mutagenesis Kit (Stratagene). The primers used for the site-directed mutagenesis reaction were **AtAox1SDMF** (5’-CGTGATGTTGTGATGGTTGTTCTGCTGGACGGCTCATCACC-3’) and **AtAox1SDMR** (5’-GGTGATGAGCCTGTCAGCACGAACAACCATCACAACATCAG-3’). The corrected codon-optimized *AtAOX1A* was removed from pUC19 using SmaI and SstI, and placed under control of root-specific patatin promoter in a pBI121-based binary vector to generate 3D-AtAox1A CO (Siritunga, 2002; Ihemere, 2003, Figure 3A). After subcloning into the binary vector, the insert *AtAOX1A* as well as the cloning sites in the
vector was fully sequenced in both directions for verification. The plasmid was introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation based on the protocol from Invitrogen (www.invitrogen.com). To 2 µL of plasmid DNA, 20 µL of thawed competent *Agrobacterium* LB4404 were added. The cell/DNA mixture was pipetted into a 10 mm cuvette and electroporated using the Biorad MicroPulser™ with voltage set at 2.2 kV. After the electroporation, 1 mL of room temperature YM medium (Invitrogen, www.invitrogen.com) was added to the electroporated DNA/cell mixture and transferred to a 15 mL culture tube for incubation at 30°C and 225 rpm for three hours. A hundred microliters of the transformation was plated on YM plates with 50 mg/L kanamycin and 30 mg/L streptomycin at 28°C for 48 hours. Plasmids were isolated from the transgenic *Agrobacteria*, and the insert was confirmed by PCR.

**Cassava transformation**

Somatic embryogenesis was induced using the method described by Ihemere (2003). Leaf lobes from 3-4 week old *in vitro* cassava plants, variety TMS 60444, were cultured on MS medium containing 8mg/L 2.4-dichlorophenoxyacetic acid (2.4-D) for induction of embryogenesis. Embryogenic callus developed within four weeks of incubation at 28°C and reduced light. Embryos were picked and plated on MS with 0.5 mg/L benzyl aminopurine for regenerating plants. Embryo cotyledons developed within four weeks on this medium. The embryo cotyledons were cut into discs and inoculated with *Agrobacterium tumefaciens* strain LBA4404 carrying the construct by placing a drop of the culture on each cotyledon. *Agrobacterium* and the plant tissue were co-cultivated for 2 days in MS medium supplemented with 8 mg/L 2.4-D, after which the cotyledons were moved to MS containing 500 mg/L carbenicillin to remove *Agrobacterium*. They were kept on this medium for two days and then transferred to MS with 8mg/L 2.4D, 500 mg/L carbenicillin and 30 mg/L paromomycin (as selection for successful transformation).

Putatively transgenic embryos were cultured on MS medium containing 1.0 mg/L benzyl amino purine (BAP). Plantlets were recovered within four weeks on this medium. Regenerated plantlets were subcultured into regular MS medium to allow development into plants. Cassava transformation using friable embryogenic callus was done following the method described by Taylor et al., (1996). Young leaf lobes were cultured on MS
medium containing 12 mg/L picloram to generate friable embryogenic callus (FECs). The FECs were co-cultivated with Agrobacterium carrying the construct for 2 days at 28°C. Subsequently, they were washed with MS liquid medium containing 500 mg/L carbenicillin to remove Agrobacterium. The washed FECs were transferred to solid medium (MS with 500mg/L carbenicillin) and kept on this medium for one week before transfer to selection medium (MS with 500mg/L carbenicillin and 30mg/L paromomycin). During a 3-6 week period, embryos were picked from this medium into regenerating medium (MS with 1 mg/L BAP). Plantlets developing from this medium were cultured on regular MS medium for further propagation and analysis.

**RT-PCR analysis of transgenic plants**

RNA was isolated from 100 mg of cassava roots using the Qiagen RNeasy Plant Mini kit (Qiagen Inc., Valencia, CA). Concentrations of RNA were measured on a spectrophotometer at 260 nm. Prior to cDNA synthesis, the RNA was treated to remove DNA contamination using the Promega DNAse treatment (Promega Corporation, Madison, WI). About 2-10 µg of RNA were used for cDNA synthesis using the qscript cDNA kit (Quanta Biosciences; MD). The cDNA was used to check for the expression of the transgene. The primers used were X0329F (GGATTAAGGCTCTTCTTGAGGAAGCA) and Nos0329R (GCCAAATGTTTG AACGATCGG), and they amplified a 500 bp region from the end of the AOX1A gene to the beginning of the NOS terminator. Tubulin primers TubF (TATATGGCCTAAGTGCGATCCTCGACA) and TubR (TTACTCTTCTGCTCAATCTCTTCTCAAGGG) were used as positive controls for the PCR reaction. The PCR reaction was based on Choice™ Taq DNA polymerase (Denville Scientific Inc.; www.densci.com). The reaction mixture contained Choice™ buffer (5 µL of 10X ), 1 µL of 10mM DNTP, 1 µL of Choice™ Taq, 0.2 µM of each of the forward and reverse primers, 2-10 µg of template DNA and deionized water to a total volume of 50 µL. The reactions for both the transgene primers (X0329F and Nos0329R) and the tubulin primers (TubF and TubR) were run with a denaturation temperature of 95°C, an annealing temperature of 52°C and an extension temperature of 72°C in the Biorad MyCycler thermal cycler (Bio-Rad Laboratories, [http://www.bio-rad.com](http://www.bio-rad.com)). Thirty-two reaction cycles were used.
**Plant growth in the greenhouse**

Cassava plantlets from tissue culture were transferred to greenhouse conditions for further analysis. Greenhouse plants were grown on the Fafard potting mix, a mixture of peat moss, perlite and vermiculite. To speed up the development of tuberous roots, plants were grown in the 3 Kord Traditional Square Pot (Kord Products, Canada). The pots have a volume of 230 mL and the small size promotes the rapid development of tuberous roots that can be analyzed within 4 months. After 4-6 months, tuberous roots were harvested from these plants and analyzed.

**Field trials**

Approximately 12 plants each of 3 transgenic lines (PAOX2-4) and 12 plants of the wild-type (Mcol 60444) were planted on June 14th, 2010 in Mayaguez, Puerto Rico and harvested on June 14th, 2011. The trial had 3 reps with 4 plants per line per rep. The entire trial plot was surrounded by 1 border row of non-transgenic plants to reduce edge effects. Total top stem length and root fresh weight and dry weight (freeze dried) yield were determined at time of harvest.

**Determination of the alternative oxidase capacity in cassava roots**

Mitochondria were isolated from cassava roots using the method described by Millar et al. (2007) with some modifications. All procedures were done at 4°C. Twenty grams of root tissue were washed and cooled to 4°C before being homogenized in a pre-chilled buffer containing; 300 mM sucrose, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 8 mM cysteine, 2% (w/w) polyvinylpyrrolidone (PVP) and 0.1% (w/w) bovine serum albumin using the Magic Bullet MB1001 blender (Homeland Houseware LLC) for 5x2 seconds. The homogenizing buffer was added at a ratio of 5 mL per g fresh weight of tissue. The suspension was filtered through 4 layers of cheesecloth and the filtrate was collected in a pre-chilled beaker on ice. The filtered homogenate was centrifuged at 3,000 g at 4°C for 5 min to remove starch and cell debris. The supernatant was then centrifuged at 15,000 g for 15 minutes and the pellet was resuspended gently using a clean soft paint brush in 5 mL of wash buffer containing 10 mM Tris-HCl pH 7.5, 300 mM sucrose, 2 mM EDTA.
and 0.1% bovine serum albumin. The resuspended solution was adjusted to 40 mL by adding more wash buffer and centrifuged at 1,000 g for 5 min. The supernatant was centrifuged at 15,000 g for 15 min and the mitochondrial pellet resuspended in 5 mL of wash buffer and kept on ice. Mitochondrial protein was determined by Bradford method. Since the extraction buffer included BSA, protein determination in the samples was estimated from similar root extractions in a buffer without BSA. To determine the alternative oxidase capacity, oxygen consumption was measured polarographically in the Hansatech oxygen electrode (model Oxygraph) as described by Bhate and Ramasarma (2010) with some modifications. All assays were conducted in 1.0 mL of standard reaction medium (10 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 mM KCl, 10 mM KH₂PO₄) using approximately 0.2 mg of protein. The respiratory capacity of AOX (alternative capacity) was measured by adding 20 mM of succinate and 1.0 mM ADP (all concentrations final). Pyruvate (5 mM) and DTT (10 mM) were added to ensure that AOX was activated. Selective inhibition of the cytochrome C oxidase pathway was achieved by addition of 1.0 mM potassium cyanide while selective inhibition of alternative oxidase was achieved by addition of 2 mM salicylhydroxamic acid (SHAM). Alternative oxidase capacity was defined as the rate of oxygen consumption that was insensitive to 1.0 mM potassium cyanide and sensitive 2 mM SHAM.

**Evaluation of postharvest physiological deterioration in cassava roots**

Postharvest physiological deterioration was evaluated using the method described by Morante et al. (2010) with some modifications. Cassava plants were harvested carefully to avoid physical damage. They were stored on plastic laboratory weighing boats at room temperature. Evaluation of postharvest physiological deterioration was done every 7 days up to 35 days. Harvested roots were cut into equal cross sections and scored for vascular discoloration using ImageJ image processing and analysis software (http://rsb.info.nih.gov/ij/). PPD was also evaluated for plants grown for 12 months under field conditions in Puerto Rico. For field grown roots, 14 cm long section was cut from the center of each root. One end of the cut section was covered with plastic wrap and the other end was left exposed to the air. The roots were placed in a growth chamber at 25 °C.
and 80% humidity. The first evaluation of post-harvest deterioration was done after 5 days with additional evaluations at 10 day intervals. Each section was cut into smaller slices of equal depth and the vascular discoloration was scored using the software ImageJ.
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Figure Legends:

Figure 1. ROS production is reduced in low cyanogen cassava plants. ROS production in high (wild type) and low (cab1-1-3) cyanogen plants. ROS accumulation is reduced in low cyanogen (Cab1) transgenic cassava lines. A) Hydrogen peroxide accumulation was determined by staining with 3,3 diaminobenzidine (DAB) and detected microscopically. B) ROS accumulation was detected using the fluorescent dye H2DCF-DA and imaged using a Zeiss LSM 510 laser confocal microscope B). All transgenic low cyanogen cassava lines (Cab1-1, Cab1-2 and Cab1-3) had significantly lower levels of ROS accumulation at p ≤ 0.05. Statistical analysis was carried out by one-way ANOVA with Dunnett’s Multiple Comparison Test.

Figure 2. Cyanogenesis induces ROS accumulation in cassava roots. A) Biochemical complementation of low cyanide plants with 5 mM potassium cyanide (KCN) results in increased ROS production in 4 weeks-old in vitro, low cyanogen (Cab1-1 to 3) transgenic plants. In vitro roots were stained with H2DCF-DA and analyzed by laser confocal microscopy. Quantification of fluorescence was done using ImageJ image processing software. The data are averages of four experiments. Error bars show 95% confidence interval. B) Inhibition of the plasma membrane NADPH oxidase. 100 µM DPI, an inhibitor of the plasma membrane NADPH oxidase, does not substantially reduce ROS production in 4 weeks-old in vitro cassava, suggesting that the ROS may be of mitochondrial origin. Fluorescence intensity was scored from images of 3 experiments using ImageJ. The data was analyzed by t-tests using GraphPad Prism software package (version 5). There was no significant difference between treated and untreated roots in treatment B at p ≤ 0.05. Error bars show confidence interval at p ≤ 0.05.

Figure 3. Expression of Arabidopsis alternative oxidase in greenhouse grown transgenic cassava roots. A) Plasmid construct of alternative oxidase. The codon-optimized Arabidopsis alternative oxidase, AtAox1A was cloned into pBI121 based-3D vector in which the CaMV 35S promoter was replaced by the root-specific patatin promoter followed by the NOS terminator (Siritunga and Sayre, 2003; Ihemere et al., 2006). B) Alternative oxidase expression in roots of transgenic lines as determined by RT-PCR. RNA was extracted from four-week old in vitro lines. Primers specific to the end of AOX1A and the beginning of the nos terminator were used to verify presence of transgene. Expression was normalized by α-tubulin primers. The expected 500 bp band for the AOX transgene was seen in PAOX1-7 and not in the wild-type. C) Alternative oxidase activity in roots of wild-type (WT) and transgenic plants overexpressing AOX. The data (nmol O2/mg protein/min) are averages of three experiments. Data analysis was by one-way ANOVA with Dunnett’s Multiple Comparisons test. Error bars show 95% confidence interval. All transgenic lines were significantly different from the wild-type at p ≤ 0.05.

Figure 4. Overexpression of alternative oxidase reduces hydrogen peroxide and ROS accumulation in cassava roots. A) roots were exposed to 3, 3 Diaminobenzidine (DAB) and imaged using a Olympus DP20 light microscope. B) roots were exposed to 2′, 7′-dichlorofluorescein diacetate (H2DCF-DA) and imaged using a Zeiss LSM 510 laser confocal microscope.
Figure 5. Expression of Arabidopsis alternative oxidase reduces onset of PPD. A) 14 days and, B) 21 days after harvest. C) PPD scores at 21 days were obtained using imageJ image processing software based on the intensity of vascular discoloration. Roots with a PPD score below 4 were considered suitable for consumption and marketing. The error bars show standard deviation. Statistical analysis was done by one-way ANOVA with Dunnett’s Multiple Comparison Test. All transgenic lines were significantly different from the wild type at p≤0.05.

Figure 6. Delayed PPD phenotype and reduced biomass was observed in some AOX transgenic lines from field trials. A) Root cross-sections were made every 2 cm. B) Three transgenic lines were used in the field trials and analysis was done 5 and 10 days after harvest. C) Storage root weight was determined from wild-type and PAOX plants grown in the field in Puerto Rico for 12 months. (WT = wild type, E = environmental exposed end of root) (n=3 from three different plants of the same line, error bars indicate standard error, an * indicates statistically different as determined by a p-value < 0.005 in relation to wild type).

Figure 7. The mechanism and control of postharvest physiological deterioration in cassava roots. Mechanical damage that occurs during harvesting operations initiates cyanogenesis by bringing linamarin and linamarase in contact. Cyanide (HCN) inhibits complex IV in the mitochondrial electron transfer chain. Inhibition of complex IV causes a burst of reactive oxygen species (ROS) production (shown as red bursts) at complexes I and III. This oxidative burst causes PPD. Overexpressing the mitochondrial alternative oxidase (AOX), which is insensitive to cyanide, prevents overreduction of complexes I and III, thus lowering ROS production and delaying PPD. Reduction of ROS to control PPD can also be achieved by overexpression of ROS scavengers (light blue dashed arrow). (HNL = hydroxynitrile lyase).
Table 1. Stem length and fresh weight in greenhouse grown (4 months) transgenic AOX plants. Stem length in meters and root tuber fresh weight in grams were measured to determine the effect of AOX overexpression on yield parameters in cassava. Statistical analysis was done by one-way ANOVA with Dunnett’s Multiple Comparison Test. Asterisks (*) indicate significant difference from the wild-type (WT).

|                | Stem Length in m | Root tuber fresh weight g per plant |
|----------------|------------------|-------------------------------------|
| WT             | 0.89±0.03        | 17.3±7                              |
| PAOX1          | 0.84±0.07        | 22±6                                |
| PAOX2          | 0.83±0.06        | 37.8±7*                             |
| PAOX3          | 0.80±0.08        | 33.2±10*                            |
| PAOX4          | 0.91±0.07        | 43.5±10*                            |
| PAOX5          | 0.76±0.06*       | 35±7*                               |
| PAOX6          | 0.67±0.08*       | 51.2±6*                             |
| PAOX7          | 0.74±0.09*       | 31.7±4.9*                           |
**Figure 1.** ROS production is reduced in low cyanogen cassava plants. ROS production in high (wild type) and low (cab1-1-3) cyanogen plants. ROS accumulation is reduced in low cyanogen (Cab1) transgenic cassava lines. A) Hydrogen peroxide accumulation was determined by staining with 3,3 diaminobenzidine (DAB) and detected microscopically. B) ROS accumulation was detected using the fluorescent dye H$_2$DCF-DA and imaged using a Zeiss LSM 510 laser confocal microscope B). All transgenic low cyanogen cassava lines (Cab1-1, Cab1-2 and Cab1-3) had significantly lower levels of ROS accumulation at $p\leq 0.05$. Statistical analysis was carried out by one-way ANOVA with Dunnett’s Multiple Comparison Test.
Figure 2. Cyanogenesis induces ROS accumulation in cassava roots. A) Biochemical complementation of low cyanide plants with 5 mM potassium cyanide (KCN) results in increased ROS production in 4 weeks-old in vitro, low cyanogen (Cab1-1 to 3) transgenic plants. In vitro roots were stained with H$_2$DCF-DA and analyzed by laser confocal microscopy. Quantification of fluorescence was done using ImageJ image processing software. The data are averages of four experiments. Error bars show 95% confidence interval. B) Inhibition of the plasma membrane NADPH oxidase. 100 µM DPI, an inhibitor of the plasma membrane NADPH oxidase, does not substantially reduce ROS production in 4 weeks-old in vitro cassava, suggesting that the ROS may be of mitochondrial origin. Fluorescence intensity was scored from images of 3 experiments using ImageJ. The data was analyzed by t-tests using GraphPad Prism software package (version 5). There was no significant difference between treated and untreated roots in treatment B at $p \leq 0.05$. Error bars show confidence interval at $p \leq 0.05$. 
Figure 3. Expression of Arabidopsis alternative oxidase in greenhouse grown transgenic cassava roots. A) Plasmid construct of alternative oxidase. The codon-optimized Arabidopsis alternative oxidase, AtAox1A was cloned into pBI121 based-3D vector in which the CaMV 35S promoter was replaced by the root-specific patatin promoter followed by the NOS terminator (Siritunga and Sayre, 2003; Ihemere et al., 2006). B) Alternative oxidase expression in roots of transgenic lines as determined by RT-PCR. RNA was extracted from four-week old in vitro lines. Primers specific to the end of AOX1A and the beginning of the nos terminator were used to verify presence of transgene. Expression was normalized by α-tubulin primers. The expected 500 bp band for the AOX transgene was seen in PAOX1-7 and not in the wild-type. C) Alternative oxidase activity in roots of wild-type (WT) and transgenic plants overexpressing AOX. The data (nmol O₂/mg protein/min) are averages of three experiments. Data analysis was by one-way ANOVA with Dunnett’s Multiple Comparisons test. Error bars show 95% confidence interval. All transgenic lines were significantly different from the wild-type at p≤0.05.
**Figure 4.** Overexpression of alternative oxidase reduces hydrogen peroxide and ROS accumulation in cassava roots. A) roots were exposed to 3, 3 Diaminobenzidine (DAB) and imaged using a Olympus DP20 light microscope. B) roots were exposed to 2’, 7’-dichlorofluorescein diacetate (H$_2$DCF-DA) and imaged using a Zeiss LSM 510 laser confocal microscope.
Figure 5. Expression of Arabidopsis alternative oxidase reduces onset of PPD. A) 14 days and, B) 21 days after harvest. C) PPD scores at 21 days were obtained using imageJ image processing software based on the intensity of vascular discoloration. Roots with a PPD score below 4 were considered suitable for consumption and marketing. The error bars show standard deviation. Statistical analysis was done by one-way ANOVA with Dunnett’s Multiple Comparison Test. All transgenic lines were significantly different from the wild type at $p \leq 0.05$. 
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