# Post-harvest physiological deterioration of cassava

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## Abstract

Within 48 hours of harvesting cassava roots undergo a rapid post-harvest physiological deterioration (PPD) that renders them unpalatable and unmarketable. PPD is a major constraint to the development of cassava for producers, processors and consumers. Deterioration is observed as blue-black vascular streaking and does not involve microorganisms. Physiologically and biochemically, PPD exhibits strong parallels to wound responses observed in other plants. However, the wound healing that normally seals the wound site and down-regulates the signals that trigger the cascades of wound responses, appears to be inadequate. As a result the wound response is not localised to the wound site in the cassava root, but is systemic throughout the root; it is this that is observed as PPD in cassava. In order to approach an understanding of PPD we have undertaken a molecular, biochemical and genetic dissection of the deterioration response. Quantitative and qualitative changes in the accumulation of several secondary metabolites have been observed. A range of cDNA clones has been isolated from PPD-related library. These clones have been sequenced and their expression patents during PPD and other cassava stress responses have been assayed in Northern blots. Genomic clones corresponding of cDNA clones have also been isolated and the expression of promoter constructs has been tested in transgenic plants. Using RFLPs genes have been included on the molecular map of the cassava genome, and used to identify quantitative trait loci for QTLs.

**Keywords:** Cassava, β-glucuronidase, gene expression, phenylalanine ammonia-lyase, post-harvest deterioration

## Introduction

Cassava (*Manihot esculenta* Crantz) is grown throughout the humid tropics from Latin America to Africa and Asia, principally for its large starchy storage roots. The roots provide the staple food for over 500 million, and in 1991 world production was 162 million tonnes; it is of particular importance to populations in sub-Saharan Africa (Wenham 1995). Cassava has the ability to grow on impoverished and marginal soils with the minimum of technological input. As a result it is often the food of the poor and can play a major role as a famine reserve crop. However, cassava is valued as a starchy component in the diet by all social strata. In addition, cassava is increasingly being grown and processed as animal feed for export, or processed industrially into a range of products including starch (Cock 1985).

Within 48 hours of harvesting the roots of cassava suffer an abiotic stress-response know as post-harvest physiological deterioration (PPD) (Beeching et al. 1998). This response renders the roots unpalatable and unmarketable. With increasing distances between farmers and markets due to urbanization, PPD has become a major constraint to the development of cassava for farmers, processors and consumers. Therefore, research directed towards introducing resistance to PPD, or delaying the response, is considered a priority by international bodies such as the FAO and the Cassava Biotechnology Network (Anonymous 1994; Wenham 1995). Increasing the shelf-life of cassava to one or two weeks, either by means of conventional breeding or via biotechnology, is considered an achievable long-term objective by these bodies. Such genetic improvement of cassava would impact positively on all concerned with its production, processing and consumption. It would particularly benefit the sustainable livelihoods of small-scale rural farmers and contribute towards poverty alleviation.

PPD is a response of the cassava root to the wounds induced during the harvesting process and is not due to microbial action, although microbial deterioration can set in subsequently (Booth 1976; Noon and Booth 1977). Visually, PPD is observed as a blue fluorescence under UV light and a blue-black streaking of the vascular tissues. In addition, coloured occlusions and tyloses from the adjacent parenchyma are seen to block xylem vessels (Rickard et al. 1979). A range of secondary metabolic products accumulate during PPD, including hydroxycoumarins, flavan-3-ols, lipids, steroids and diterpenoids (Lalaguna and Agudo 1989; Rickard 1981; Sakai and Nakagawa 1988; Tanaka et al. 1983). Of particular interest is the

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hydroxycoumarin, scopoletin, which is especially abundant during PPD and is largely responsible for the blue fluorescence seen under UV light. Application of exogenous scopoletin has been shown to accelerate PPD symptoms (Wheatley and Schwabe 1985). Scopoletin, and other PPD-induced secondary metabolites, show anti-microbial activities (Giesemann et al. 1986; Rodriguez et al. 2000; Taniguchi et al. 1984).

During PPD the cassava root is biochemically active. PPD is accompanied by increases in respiration in the root and some mobilisation of starch into sugars (Hirose 1986; Uritani et al. 1984). The phytohormone ethylene, which is known to co-ordinate wound and senescence responses in other plants (Ecker and Davis 1987), also increases in the deteriorating cassava root (Hirose et al. 1984), and a cDNA for ACC oxidase, the last enzyme in the biosynthesis of ethylene, has been cloned from a PPD-related library (Li et al. 2000). Various enzymes, including phenylalanine ammonia-lyase (PAL), catalase, peroxidase, dehydrogenase and polyphenol oxidase, increase in activity during PPD (Czyhrinciw and Jaffé 1951; Hirose 1986; Plumbley et al. 1981). It is probable that the interaction between some of these enzymes and secondary metabolites is responsible for the discoloration observed during PPD. Evidence from inhibition by cyclohexamide, *in vivo* labelling of proteins and cDNA cloning confirm that PPD is an active process involving changes in gene expression and protein synthesis (Beeching et al. 1995; Beeching et al. 1997; Uritani et al. 1984).

The changes observed in cassava roots during PPD closely resemble important aspects of the wound response in other, more fully studied plant systems (Bennett and Wallsgrove 1994; Bowles 1990; Bowles 1998). The initial wounding of a plant releases signals that trigger responses in the plant. Some of these signalling molecules are produced by the act of wounding itself, such as cell-wall fragments or lipid peroxidation products, whilst others are released from inactive precursors or synthesised *de novo*, such as jasmonic acid, salicylic acid, ethylene, systemin or $H_2O_2$. These signals either act locally to the wound site to induce defensive and protective changes, or systemically in the plant to prepare the plant for the possible extension of the wounding and potential pathogen invasion. The principal responses of the plant to these signals include the production of enzymes and secondary metabolites that have a defensive, anti-microbial, role, such as glucanases and chitinases, phytoalexins and anti-oxidants; and the synthesis of molecules involved in wound repair, these include, callose, lignin, suberin and the insolublisation of hydroxyproline-rich glycoproteins (HRGPs) by $H_2O_2$. The repair and sealing of the wound site by these compounds has the effect of removing the source of the signals that triggered the wound response in the first place, thereby down-modulating that response and returning the plant to normal development. While many of these wound response elements, which are observed in other plants, are present in the cassava root during PPD, the wound repair aspects are inadequate or lacking. Therefore, the production of the signals that initiate the wound response in cassava is not switched off, thereby triggering a continual cascade of wound responses throughout the root, which is observed as PPD.

If the cassava root remains attached to the plant, wounds are repaired. In addition, conditions such as high temperature and humidity, and exclusion of oxygen will induce suberisation and inhibit PPD in the detached roots (Booth 1976; Rickard and Coursey 1981). These data imply that cassava root does have the capacity to repair wounds, but that this is inadequate once harvested. Unlike many other root crops, the cassava storage root is not a propagule, it solely functions as a store of photosynthate for the plant. Therefore, as the storage root has no function once detached from the plant, there is no biological need for it to be able to repair wounds under such circumstances. However, variation in the PPD response of cassava is found within germplasm collections, implying that this response is under genetic control, although it is also influenced by environmental parameters (Iglesias et al. 1994).

The observations that the initial visual symptoms of PPD occur in the vascular tissue of the root and that the exclusion of oxygen prevents this deterioration, suggest that the entry of oxygen into the root *via* the vascular system, coupled with the concomitant loss of water, in some way triggers the response. The implication being that PPD is an enymatically mediated oxidative process (Marriott et al. 1978; Noon and Booth 1977; Plumbley and Rickard 1991). Certainly, the oxidation of secondary metabolites is well known to cause wound-induced discoloration in harvested plant materials (Salveit 1997). Oxygen, and the reactive oxygen species (ROS) into which it can be converted in the plant, have been implicated in a range of plant stress responses from wounding to pathogenesis (Baron and Zambryski 1995). While some ROS, particularly $H_2O_2$, can play a positive role during wound responses in lignin biosynthesis, HRGP cross-linking and signalling, others can potentially damage the plant. As a result the plant possesses a battery of enzymes, such as superoxide dismutase, catalase and peroxidase, and compounds such as phenolic anti-oxidants, to modulate ROS. Several of these enzymes and compounds increase in abundance during cassava PPD, providing further circumstantial evidence for the role of ROS in PPD. In addition, the powerful anti-oxidant, β-carotene tends to be positively correlated with reduced PPD response (Iglesias et al. 1995).

Many of the changes occurring during PPD derive from general phenylpropanoid metabolism, of which the enzyme phenylalanine ammonia-lyase (PAL) catalyses the first committed step. The products of general phenylpropanoid metabolism include anti-microbial compounds, anti-oxidants, signalling, and wound-healing compounds (Dixon and Paiva 1995; Hahlbrock and Scheel 1989). The activities of PAL, and its gene(s), peak during the first days of PPD. Due to its importance in plant stress responses, PAL has been the subject of intensive study in many plants.

However, its role and importance during cassava PPD is largely unknown.

In this paper we examine the role of enzymes and metabolites that play key roles in modulating ROS during PPD and other stress responses, and in particular a gene for PAL that is active during development and PPD.

## Methods

Cassava plants were grown either in the field at CIAT, Cali, Colombia, or in the tropical glasshouse at Bath. Cassava cell suspension cultures (cultivar MCOL 22) were grown on liquid MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose and 2 $mgl^{-1}$ 2,4-dichlorophenoxyacetic acid. The cultures were maintained by shaking in an orbital incubator in the dark at 110 rpm at 25°C, and sub-cultured every seven days. Glucan cell-wall elicitor was prepared from baker's yeast (Schumacher et al. 1987).

Hydrogen peroxide in cassava root slices was detected histochemically by light microscopy (Olsen and Varner 1993), localised by 3,3-diaminobenzidene tetrahydrochloride (DAB) vacuum infiltration (Vallélian-Bindschelder et al. 1998), and quantified (Warm and Laties 1982).

Ethanolic extracts of cassava roots were separated on HPTLC plates (silical gel 60 $F_{254}$, 20×20, Merck) using a liquid phase of chloroform : ethyl acetate : methanol (2:2:1), and anti-oxidants were visualised under UV light after spraying with 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Takao et al. 1994). HPLC was carried out using a Gilson system combined with a diode array detector (Hewlett Packard) and an analytical reversed phase column (Techsphere ODS-BDS, 250×4.6mm; 5µm; HPLC Technology, UK), using aqueous $H_3PO_4$ (pH 2.6) in a linear gradient of two to 100% acetonitrile.

RNA was extracted using the method of Chang et al. (1993). Other methods were as Sambrook et al. (1989).

Transgenic cassava was produced as Schopke et al. (1996) and β-glucuronidase was detected according to Jefferson et al. (1987).

## Results

### Modulation of reactive oxygen species production during stress responses.

DAB infiltration of cassava root slices over a time course after harvest revealed that in cultivars with a low or intermediate PPD response (NGA 2, MDOM 5 and CMC 2177-2) showed an intense colour reaction associated with $H_2O_2$ accumulation which peaked at 24 hours after harvest. In contrast, in the highly susceptible cultivar, MCOL 22, this peak occurred later, after three days. Quantification of $H_2O_2$ confirmed these findings. Histological observation showed that $H_2O_2$ first accumulated in the xylem parenchyma by 24 hours after harvest, subsequently was detected in the storage parenchyma, and, in some cultivars, also in the cortical parenchyma. In all cases, the $H_2O_2$ was detected in the apoplast of the cells, especially in the area of the middle lamella.

In the cassava cell suspension culture model system challenged with either elicitors or micro-organisms peaks of $H_2O_2$ were also observed. A range of fungal-derived elicitors produced peaks within 20 minutes of elicitation (Figure 1). However, in the case of co-culture with a range

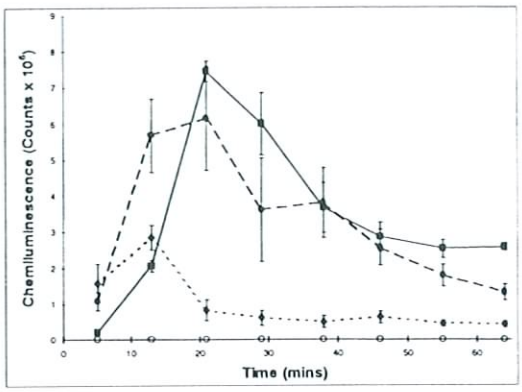


**Figure 1**
Oxidative burst of $H_2O_2$ in cassava cell suspension cultures in response to a range of elicitors. ◆- oligogalacturonic acid (7.5 µg/ml), ■- *Colletotrichum lindemuthianum* glucan cell-wall (18 µg/ml), ●- baker's yeast glucan cell-wall (50 µg/ml), ○- control

of micro-organisms, in addition to an early peak at 20 – 30 minutes, a second was observed in response to the incompatible pathogens, *Erwinia amylovora* and *Pseudomonas syringae* at 2 hours after inoculation (Figure 2). In the case of the former, the second peak of $H_2O_2$ was substantially larger than the first peak. On the other hand, in the case of the compatible pathogen, *Xanthomonas axonopodis* p.v. *manihotis*, no secondary peak was observed.

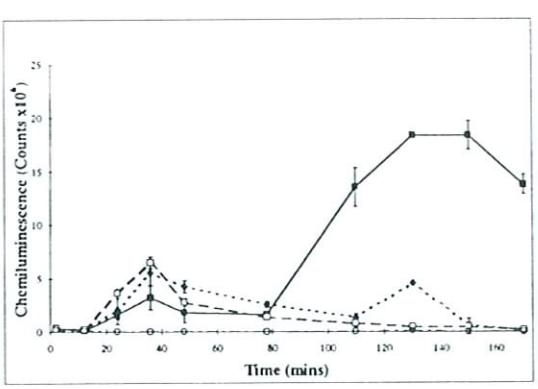


**Figure 2**
Oxidative burst of $H_2O_2$ in cassava suspension cultures co-cultivated with micro-organisms. ■- *Erwinia amylovora*, ◆- *Pseudomonas syringae*, □- *Xanthomonas axonopodis* p.v. *manihotis*, ○- control

In addition to reactive oxygen species (ROS) being produced in cassava in response to the stresses of PPD, elicitation or microbial infection, genes for enzymes and proteins that modulate or use ROS were activated. Northern blots of RNA isolated from cassava roots during PPD of from cell suspension cultures elicited with yeast glucan cell-wall elicitor were probed with cassava cDNA clones for catalase, peroxidase and hydroxyproline-rich glycoprotein (HRGP). In all cases gene expression increased during the stress responses compared to the controls.

Secondary metabolites with anti-oxidant properties were detected in cassava roots during a time course of PPD using HPTLC plates sprayed with DPPH, indicating that these increased during the PPD response (Figure 3). These included the flavan-3-ols, (+)-gallocatechin, (+)-catechin and (+)-catechin gallate, identified by HPLC. While these three compounds are powerful anti-oxidants, they peaked in abundance after day four post-harvest, by which time the visible symptoms of PPD were already very apparent.

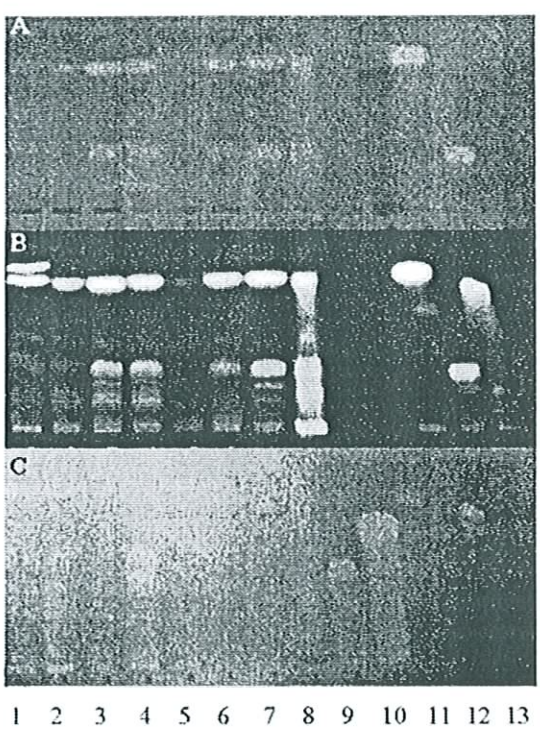


**Figure 3**
Detection of anti-oxidant compounds that accumulate during PPD in the cassava root. Separation of ethanolic extracts by HPTLC. A – viewed at 254 nm UV light; B – viewed at 366 nm UV light; C – sprayed with DPPH to detect anti-oxidants and viewed under normal light. Lane 1– MCOL 22 day 0; 2– day 1; 3– day 4; 4 – day 6 after harvest; lane 5 – NGA 2

However, the hydroxycoumarin scopoletin, which is a less powerful anti-oxidant, showed an increase in abundance earlier; it was readily detectable in the roots within 24 hours of harvesting.

Crude extracts from cassava roots undergoing PPD gave a blue-black precipitate when scopoletin and $H_2O_2$ were added, which did not occur when other secondary metabolites produced during PPD were used (Figure 4). An identical response was observed when horse-radish peroxidase was used instead of the cassava extract. These data suggest that the blue-black discoloration of the cassava roots vascular tissue during PPD could be due to this reaction.

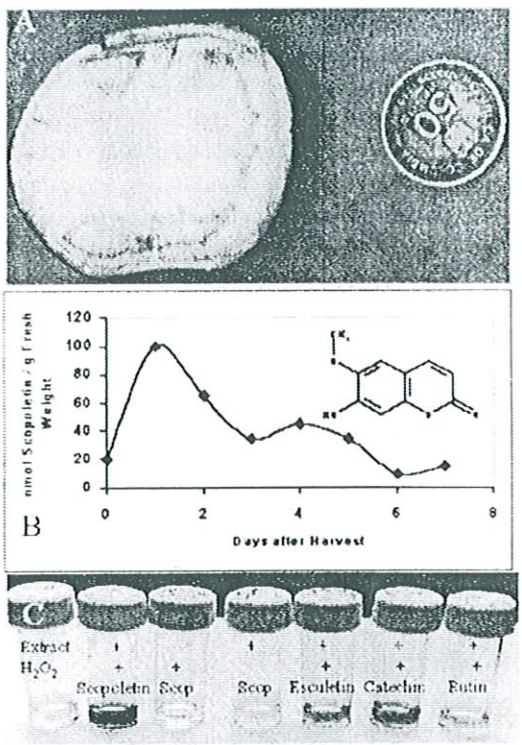


**Figure 4**
Cassava root extracts complex scopoletin with $H_2O_2$ to form an insoluble blue-black precipitate. A – slice of cassava storage root undergoing PPD, B – changes in abundance of scopoletin in cassava roots during PPD, C – only scopoletin forms a blue-black precipitate with $H_2O_2$ and root extract

### Expression of phenylalanine ammonia-lyase in cassava

Cloning and Southern hybridisation experiments indicated that cassava contains at least three different phenylalanine ammonia-lyase (PAL) genes. Northern hybridisation showed that PAL mRNA accumulates and peaks in cassava roots during the PPD time course and during the response of cassava cell suspension cultures to yeast glucan cell-wall elicitor. A clone for PAL, gMePAL2, was isolated from a cassava genomic library and sequenced. This clone consisted of a transcribed region containing a single intron at a site conserved between PALs from other plant species and approximately 900 bp of the promoter region. 840 bp of this PAL promoter was cloned in front of the β-glucuronidase gene (GUS) and co-transformed into cassava

embryogenic suspension cultures using particle bombardment.

Although transgenic callus strongly expressed GUS throughout the tissue, many developing embryos lost this ability (Figure 5A). Plants were regenerated from the transgenic material. In these transgenic plants GUS expression was confined to the xylem parenchyma in all tissues examined (Figure 5). However, in some tissues GUS expression was also detected in the cork cambium (Figure 5F). Both vegetative and storage cassava roots

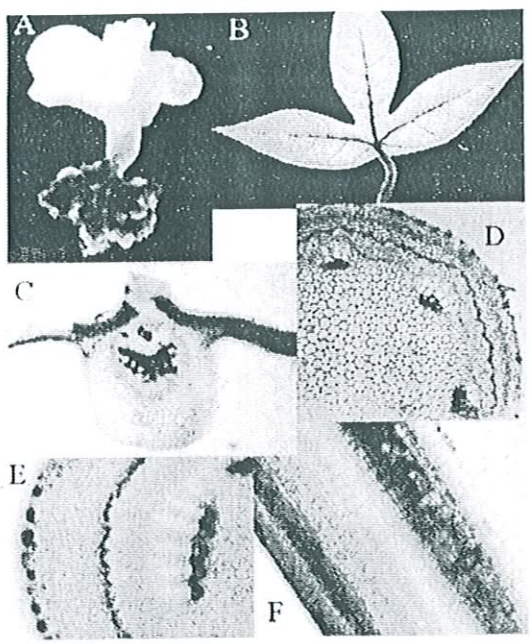


**Figure 5**
Transgenic cassava expressing GUS driven by 840 bp of the PAL2 promoter during normal development. A – callus and developing embryo, B – leaf, C – transverse section of leaf, D – transverse section of petiole, E – transverse section of stem, F – longitudinal section of stem.

similarly expressed GUS in their xylem parenchyma during normal development (Figure 6A & 6B). Perhaps due to this endogenous vascular expression of GUS it was difficult to determine visually whether or not there had been a change in GUS expression during PPD in the harvested storage roots (Figure 6C & 6D). However, preliminary *in vitro* assays indicated an increase in GUS activity during the PPD response.

## Discussion

The data presented here provide strong evidence for reactive oxygen species, especially $H_2O_2$, playing important roles during PPD and during other stress responses of cassava. The cell suspension culture and elicitor work, in particular, confirms that cassava is capable of an oxidative burst, including a secondary burst in the case of interactions with incompatible pathogens. Such oxidative bursts have been observed in other plants and plant pathogen

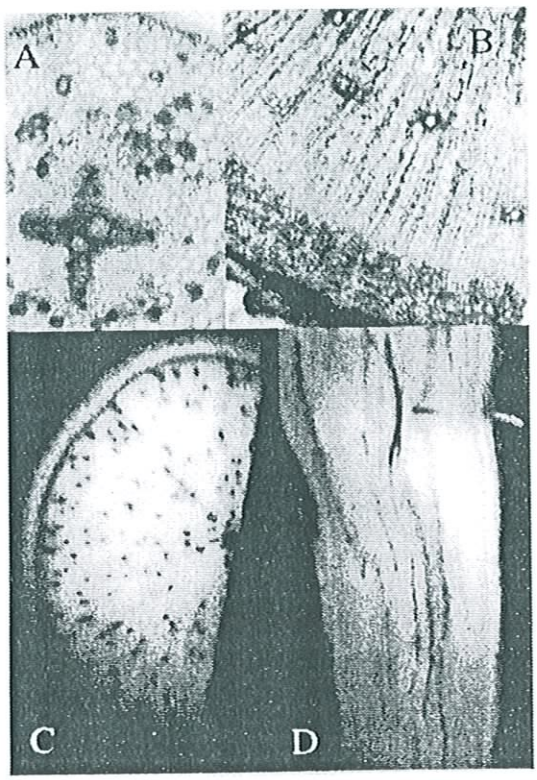


**Figure 6**
Transgenic cassava expressing GUS driven by 840 bp of the PAL2 promoter in the roots. A – transverse section of vegetable root, B – transverse section of storage root, C – transversely sliced root undergoing PPD, D – longitudinally sliced root undergoing PPD.

interactions where they can also be part of the plant's hypersensitive response to incompatible pathogens (Heiser and Elstner 1998; Low and Merida 1996). In addition to the production of $H_2O_2$, compounds that can function as anti-oxidants, enzymes that can modulate ROS, and proteins that require $H_2O_2$, accumulated in the storage root during PPD and in elicitor-challenged cell suspension cultures. These are all components of plant wound responses, thereby providing further confirmatory evidence that cassava, including the deteriorating detached root, is capable of normal wound responses. In particular, it is interesting that the expression of hydroxyproline-rich glycoprotein (HRGP) genes increases during these stress responses, as the insolublisation of HRGPs by $H_2O_2$ is part of the normal process of wound repair (Sommer-Knudsen et al. 1998). This is surprising, as effective wound repair is not observed in harvested cassava roots kept under normal conditions, yet here we have evidence that part of this repair process is expressed. Deteriorating cassava root extracts, or peroxidase, were shown to be capable of forming an insoluble precipitate with the $H_2O_2$ and the hydroxycoumarin, scopoletin. This may explain, at least in

part, the observed blue-black discoloration (vascular streaking) observed in the vascular tissues of the deteriorating roots.

Phenylalanine ammonia-lyase (PAL) catalyses the first committed step of general phenylpropanoid metabolism, the products from which are important in many aspects of normal development and in stress-related responses. Here we demonstrate that 840 bp of the MePAL2 promoter is sufficient to drive the reporter gene, GUS, and that such expression is regulated in a developmental and tissue specific manner. During development the expression of the reporter construct was exclusively observed in the xylem parenchyma of the vascular tissues and, in some tissues, also in the cambium. While the results presented require further confirmation, they indicated that the expression of the PAL promoter-GUS construct increases during the PPD response of the cassava storage root. The vascular tissue in the root in which the 840 bp promoter is active is also the site of the vascular streaking seen in the deteriorating root.

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