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Towards identifying the full set of genes expressed during cassava post-harvest physiological deterioration

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Abstract Storage roots of cassava (*Manihot esculenta* Crantz) exhibit a rapid post-harvest physiological deterioration (PPD) response that can occur within 24–72 h of harvest. PPD is an enzymatically mediated oxidative process with parallels to plant wound, senescence and defence responses. To characterise those genes that show significant change in expression during the PPD response we have used cDNA microarray technology to carry out a large-scale analysis of the cassava root transcriptome during the post-harvest period. We identified 72 non-redundant expressed sequence tags which showed altered regulation during the post-harvest period. Of these 63 were induced, whilst 9 were down-regulated. RNA blot analysis of selected genes was used to verify and extend the microarray data. Additional microarray hybridisation experiments allowed the identification of 21 root-specific and 24 root-wounding-specific sequences. Many of the up-regulated and PPD-specific expressed sequence tags were predicted to play a role in cellular processes including reactive oxygen species turnover, cell wall repair, programmed cell death, ion, water or metabolite transport, signal transduction or perception, stress response, metabolism and biosynthesis, and activation of protein synthesis.

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Abbreviations

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
ADC	Arginine decarboxylase
ADP	Adenosine triphosphate
AKR	Aldo/keto reductase
AP	Adaptor protein
ATP	Adenosine triphosphate
ARP	Auxin repressed protein
cDNA	Complementary deoxyribonucleic acid
CIAT	International Centre for Tropical Agriculture
DNA	Deoxyribonucleic acid
ELIP	Early light inducible protein
EST	Expressed sequence tag
FKBP	FK binding protein
GLP	Germin-like protein
GRP	Glycine-rich protein
GST	Glutathione-S-transferase
H^+-PPase	Proton translocating inorganic pyrophosphatase
H_2O_2	Hydrogen peroxide
HRGP	Hydroxyproline-rich glycoprotein
LEA	Late embryogenesis abundant
MIP	Major intrinsic protein
OxO	Oxalate oxidase
PCD	Programmed cell death
PCR	Polymerase chain reaction
PIP	Plasma membrane intrinsic protein
PPD	Post-harvest physiological deterioration
PPIase	Peptidyl-prolyl *cis*/*trans* isomerase

PPR	Pentatricopeptide repeat
PRP	Proline-rich protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAM	Significance Analysis of Microarrays
SIR	Sulphite reductase
SOD	Superoxide dismutase
TCTP	Translationally controlled tumour protein
TDF	Transcript-derived fragment
UBP	Oligouridylate binding protein
UDP	Uridine diphosphate
UTR	Untranslated region
XET	Xyloglucan endotransglcosylase

Introduction

Post-harvest physiological deterioration (PPD) is an endogenous root disorder affecting the storage roots of cassava (*Manihot esculenta* Crantz). Within 24–72 h of harvest, depending on the cultivar and environmental conditions, roots exhibit a blue–black discoloration of the xylem vessels known as ''vascular streaking'' and display other unpleasant organoleptic properties such as impaired flavour and odour. This rapid deterioration is a major constraint on the development of this important crop, which impacts on farmers, processors and consumers alike.

Vascular streaking initiates at wound sites caused by harvesting (Booth 1976) and is thought to result largely from peroxidase mediated oxidation of scopoletin (Tanaka *et al.* 1983; Wheatley and Schwabe 1985). Biochemical localisation data indicate that required components of the reaction, namely scopoletin, H_2O_2, and peroxidase activities, are initially localised to the vicinity of the xylem vessels where vascular streaking symptoms occur. Moreover, peroxidase isoforms with activity towards scopoletin are present in the root (Reilly *et al.* 2003). Other observed biochemical changes during PPD include increases in respiration (Hirose 1986), changes in lipid composition (Lalaguna and Agudo 1989), ethylene synthesis (Hirose *et al.* 1984), the occurrence of a wound-induced oxidative burst (Reilly *et al.* 2003), and the accumulation of secondary metabolites (Rickard 1981; Sakai and Nakagawa 1988; Uritani *et al.* 1983; Wheatley and Schwabe 1985). Secondary metabolites which accumulate during PPD include diterpenic and phenolic compounds, several of which show anti-microbial and/or anti-oxidant activity (Buschmann *et al.* 2000a, b; Sakai and Nakagawa 1988).

At the molecular level the development of PPD is evidently a complex phenomenon involving multiple components. Cycloheximide inhibition of protein synthesis indicates PPD is an active rather than degenerative process involving changes in gene expression and protein synthesis. Several analyses of genes expressed during PPD (Han *et al.* 2001; Huang *et al.* 2001; Reilly *et al.* 2001, 2003) show altered regulation of proteins and enzymes involved in signal transduction, reactive oxygen species (ROS) modulation, phytohormone synthesis, senescence and programmed cell death (PCD) responses, synthesis of anti-microbial, anti-oxidant or other defensive compounds, and the formation of other compounds that are involved in the synthesis of cell wall components, as well as proteins that are themselves components of cell walls.

Under normal storage conditions wound healing of the root is inadequate; however, harvested roots are capable of producing a wound periderm under conditions of high temperature and humidity (curing), although this occurs slower than in other root crops. It has been proposed that wound repair and subsequent down-modulation of the wound signals are impaired, resulting in cascades of wound responses that spread throughout the root (Han *et al.* 2001). Interestingly, wounded roots that remain attached to the plant are capable of normal wound repair (Mwenje *et al.* 1998). Signalling components that could lead to the modulation of PPD responses include the plant hormone ethylene (Hirose 1986) and a wound-induced oxidative burst (Reilly *et al.* 2003). The role of hydrogen peroxide as a signalling molecule in plants is becoming increasingly clear and hydrogen peroxide may function to regulate several signalling pathways. Microarray data have indicated that around 1% of the *Arabidopsis thaliana* transcriptome is regulated by hydrogen peroxide (Desikan *et al.* 2001). This approach highlights the usefulness of exploiting massively parallel methods for gene discovery and analysis. Using a microarray of 5,700 cassava expressed sequence tags (ESTs) enriched for cDNAs expressed during starch biosynthesis and bacterial blight infection this approach has been successfully exploited to study the gene expression profile in cassava challenged with *Xanthomonas axonopodis* (Lopez *et al.* 2005). An analysis of the cassava proteome has also been used to explore protein accumulation during the developing storage root (Sheffield *et al.* 2006). In this paper we employ a cDNA-microarray constructed from cassava roots over a deterioration time course as a tool for gene discovery in order to identify PPD-responsive genes in the cassava storage root.

Materials and methods

Plant material

Cassava plants were grown in the tropical greenhouse at the University of Bath at 22–28°C, relative humidity 40–80%, and a minimum light period of 12 h per day under daylight, supplemented with 400 W Phillips high-pressure sodium lights when necessary. Storage roots (cultivar CM2177-2) were harvested after 9 months growth. Alternatively, roots were obtained from field-grown plants at CIAT, Cali, Colombia. Cassava leaf tissue was obtained from greenhouse-grown plants at the University of Bath.

Plant treatments

Induction of PPD in cassava storage roots was carried out as described previously (Reilly *et al*. 2003). For the leaf-wounding treatments, leaves were injured by crushing and were either immediately detached from the plant and stored at ambient temperature, or were left attached to the plant to allow initiation of wound repair. Samples were frozen in liquid nitrogen after 24 h.

cDNA library construction

Total RNA was extracted and mRNA purified from a range of time-points over a deterioration time course from roots of cassava cultivar CM2177-2 according to previously described methods (Reilly *et al.* 2001). Purified mRNA from time-points 0, 6 and 12 h after harvest were pooled as was mRNA from 24, 48 and 96 h and used to generate ''early'' and ''late'' PPD cDNA libraries in Lambda Zap II (Stratagene). These libraries should contain cDNA copies of all the genes expressed in the mature root at harvesting and during the first 4 days post-harvest.

Preparation and hybridisation of cDNA microarrays

Individual clones from the two PPD-related cDNA libraries were amplified by PCR and spotted on to PL-100C-Poly L lysine slides (CEL Associates) using a Hitachi SPBIO robotic arrayer. For each hybridisation 11,136 randomly chosen cassava cDNAs were spotted, 7,680 from the ''early'' and 3,456 from the ''late'' libraries. In addition, the following control DNAs were included on each slide: E1 (GenBank accession U89872); E2 (AF878810); E3 (NM_000858); E5 (K01193); E6 (X65316); E7 (AF126210); E8 (X13988); E9 (M21812); E10 (X07868), as ''spike'' genes; three well characterised genes from other plants (M76402, BE341179 and EF044211; and ten cassava genes with known PPD-expression profiles (EF035080, AF170272 AF239615, AY973618, EF035081, AF265551, AF266465, AF323605, AY036011 and AY033386). On each slide, every cDNA was spotted four times in order to generate four technical replicates per slide. As targets, mRNA was purified from roots of cultivar CM2177-2 over a deterioration time course, or from injured or uninjured leaves of the same cultivar. This mRNA was used to generate cDNA hybridisation targets labelled with either Cy3 or Cy5; cDNA synthesis was as Lopez *et al*. (2005). In order to obtain statistically reliable data, three biological replicate experiments were carried out for each hybridisation using independently isolated mRNA samples.

Microarray analysis

The PPD time course microarray data were analysed using the TIGR spotfinder and MIDAS software packages within the TM4 open source suite of programs (Saeed *et al.* 2003, www.tigr.org/softlab) using Lowess (Locfit) normalisation parameters applied globally for each slide followed by standard deviation regularisation between subgrids and slides. The mean (μ) and standard deviation (σ) for the distribution of $\log_2$ (ratio) values were calculated and differential expression at the 95% confidence level was identified as $\log_2$ (ratio) values greater than 1.96 standard deviations from the mean. For significance of microarray analysis of the microarray data of the comparisons between leaves and roots, gene expression was normalised using MIDAS (Saeed *et al.* 2003) by applying Lowess intensity-dependent normalisation by subgrids, followed by a standard deviation regularisation between subgrids and between slides. Genes expressed in the roots and not in the leaves at each root vs. leaf comparison were identified using the one-class design of the significance analysis of microarrays method (Chu *et al.* 2002), selecting 5,000 permutations and a false discovery rate of 0.1% or lower. The root-expressed genes common to the three comparisons were classified as root-specific.

RNA blot analysis

Total RNA for Northern hybridisations was extracted from storage roots or leaves and blotted and hybridised as Reilly *et al*. (2001). Hybridisation was carried out

with ^{32}P-labelled cDNA probes prepared using the Ambion Stripeze labelling kit according to the specifications of the manufacturer.

Bioinformatics

Single-pass sequencing of identified cDNA clones of interest was carried out and sequence data analysed using nBLAST and tBLASTx (Altschul *et al*. 1997, http://www.ncbi.nlm.nih.gov/). Where the deduced EST sequence identities were similar, multiple alignments were carried out using CLUSTALW (Chenna *et al*. 2003, http://www.ebi.ac.uk/clustalw) to identify and exclude duplicate sequences. Prediction of theoretical pI and other sequence analyses were carried out using http://www.expasy.org. Heat maps for visualisation of expression data were generated using Cluster and Treeview (http://rana.lbl.gov/EisenSoftware.htm) programs (Eisen *et al.* 1998). EST and cDNA sequences were submitted to the NCBI database (http://www.ncbi.nlm.nih.gov) using dbEST batch submission or Bankit submission protocols respectively, and the individual accession data are given with the corresponding tables.

Results

With the aim of generating a more complete understanding of the processes that occur in the cassava storage root during PPD we have used two complementary microarray hybridisation strategies. Firstly, transcript expression levels were examined over a PPD time course. For each of the five hybridisation experiments the ''control'' Cy3-labelled target was prepared from root mRNA immediately after harvest and the ''experimental'' Cy5-labelled target from root mRNA at 12, 24, 48, 72 and 96 h after harvest, respectively. Analysis of these data would be expected to identify genes showing significant up- or down-regulation during PPD. Secondly, we used three microarray hybridisation experiments to compare expression levels between cassava roots and leaves under different conditions. For these experiments mRNA was purified from both roots and leaves at 0 and 24 h after harvest or wounding. A comparative analysis of the hybridisation of the root at 0 h after harvest vs. leaves at 0 h against root at 0 h after harvest vs. leaves at 24 h, together with roots 24 h after harvest vs. leaves 24 h after wounding, would be expected to identify potential root-specific genes, including a subset of PPD-related genes that are expressed in the root prior to harvest but which may subsequently also show differential expression. On the other hand, the comparison of root 24 h after harvest vs. leaves 24 h after wounding alone should allow identification of root injury-specific and thus potentially PPD-specific genes which show *de novo* induction in the root after injury.

The control genes included in the microarrays served two purposes: (a) as ''landing lights'' to aid correct grid alignment for the spot detection and measurement steps; and (b) to ensure correct channel allocation. For each output file we confirmed that the control genes showed expected expression and that replicates were reasonably consistent; the controls were then excluded from further analysis (data not shown).

PPD time course microarray analysis

A scatter plot of signal intensities of all 11,136 cassava root cDNA clones for each of the microarray time course experiments is shown in Fig. 1. Each spot represents the mean of 12 normalised signal intensity values (four technical replicates for each of three biological replicates). The majority of transcripts remained unchanged during the post-harvest period, with a peak time-point of differential gene expression observed at 24 h after harvest.

Based on the selection criteria described in Sect. ''Materials and methods'', transcripts showing differential expression at the 95% confidence level were identified. For the up-regulated genes, only those clones showing a consistent expression profile across the three biological replicate hybridisations were selected for sequencing. In addition, as PPD is an active process it was expected that up-regulated genes would be of greater interest with respect to PPD than down-regulated genes. Thus, for the PPD down-regulated clones, stricter selection criteria were applied; only those showing both a consistent expression profile across the three biological replicate hybridisations and with greater than twofold down-regulation were selected for sequencing.

While no specific effort was made to measure redundancy in the cDNA microarray by sequencing random clones, an impression of this can be gained by comparing the number of clones initially identified in this experiment with that of the non-redundant ESTs, discussed below. The mean representation in the array of each EST was 2.76 with a standard deviation of 3.87. This high variability was due largely to four clones that were represented ten or greater times in the initial selection. It is important to take into consideration that the clones that were selected in this paper were done so using criteria that included significant changes in gene

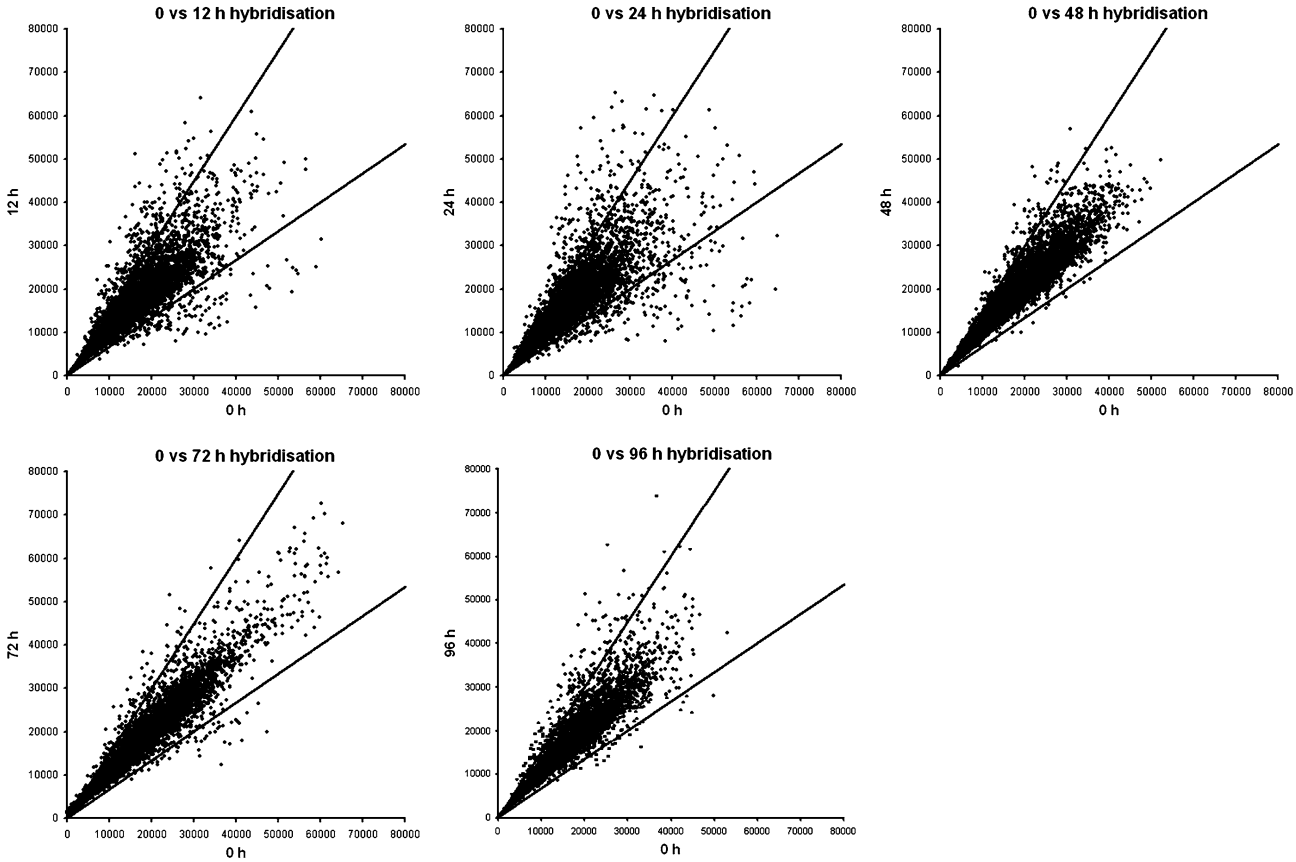

Fig. 1 Scatter plot graphs of normalised channel intensities of all ESTs on the PPD microarray for the time-point hybridisation experiments. Signal intensities for each clone are plotted with control (0 h) and experimental (12, 24, 48, 72 or 96 h after harvest) on the *X* and *Y* axes, respectively. The diagonal lines indicate 1.5-fold induction/repression ratio cut-offs

expression and as a result their representation in the microarray may not be wholly comparable to those other clones that do not fulfil these selective criteria.

Based on BLAST homology searches and a review of the literature, putative identities and possible functions were assigned and are shown in Table 1. Discounting duplicated sequences we were able to identify 63 non-redundant ESTs showing up-regulation during the PPD time course and 9 that showed down-regulation. A heat map of expression profiles for these ESTs is shown in Fig. 2. This shows nine ESTs encoding xyloglucan endotransglycolase, ribosomal protein L5, 1-aminocyclopropane-1-carboxylase (ACC) oxidase, pentatricopeptide protein, peroxidase, adenosine triphosphate/adenosine diphosphate (ATP/ADP) translocase, cytochrome b_5, heat shock protein and an unknown protein, showed strong up-regulation within 12 h after harvest. A larger group showed strong up-regulation within 24 h after harvest. Both groups may be considered as early PPD-responsive genes. ESTs corresponding to a catalase MecCAT1 (AF170272; Reilly *et al.* 2001), a hydroxyproline-rich glycoprotein MecHRGP1 (AF239615; Han *et al.* 2001), and two Cytochromes P_{450} sequences CYP79D1 and CYP79D2 (AAF27289 and AAF27290; Andersen *et al.* 2000), previously shown to be expressed in the storage root after harvest were amongst those identified as showing up-regulation in the microarray.

Reactive oxygen species turnover

It is increasingly apparent that ROS and the enzymes responsible for their interconversion are involved in a range of plant developmental and stress responses including defence, pathogenesis, PCD and senescence. A large minority of the up-regulated genes identified in Table 1 (18%) is involved in the turnover of ROS, and may function directly to detoxify harmful ROS or indirectly to utilise ROS in cellular functions. The catalases (EC 1.11.1.6), ascorbate peroxidases (EC 1.11.1.11) and secretory peroxidases (EC1.11.1.7) catalyse the reduction of H_2O_2 either directly, using ascorbate or a range of organic or inorganic substrates as an electron donor. MecCAT1 (DT883577, AF170272) was previously isolated in this laboratory and showed up-regulation in the cassava storage root

Table 1 Post-harvest physiological deterioration (PPD)-responsive and putative PPD-specific ESTs

Putative identity	EST accession no.	BLAST hit	*E*-value	Average ratio					
				0 vs. 12 h	0 vs. 24 h	0 vs. 48 h	0 vs. 72 h	0 vs. 96 h	R24 vs. L24
PPD up-regulated genes									
Roles in ROS turnover									
Catalase CAT1	DT883577	AAD50974	2e-97	0.9	1.1	1.1	1.5	1.1	1.0
Catalase CAT2 (MecCAT2, AY973614)	DN740378	AAD50974	0.0	0.9	1.5	1.1	1.6	1.1	1.3
Ascorbate peroxidase (MecAPX2, AY973622)	DN740373	AAO14118	7e-80	1.3	1.7	1.1	1.2	1.0	1.2
Secretory peroxidase (MecPX3, AY973612)[a]	DN740367	CAA09881	2e-94	1.8	1.5	0.9	1.1	1.0	1.5
Thioredoxin peroxidase	DT883578	CAC84143	4e-102	1.4	1.7	1.1	1.1	1.0	0.8
Thioredoxin-like protein	DT883579	AAG51342	1e-53	1.3	1.5	1.2	1.4	1.2	1.2
Glutathione S-transferase	DT883580	AAC18566	2e-67	2.0	2.3	1.1	1.2	1.2	1.3
Metallothionein	DT883581	AAT02525	6e-9	1.3	1.7	1.1	1.2	1.2	1.2
Quinone-oxidoreductase[a]	DT883582	AAR07601	3e-8	3.5	6.2	2.1	2.0	1.9	3.2
Aldo/keto reductase (MecAKR, AY973615)	DN740363	AAX84672	1e-111	0.7	1.1	1.1	1.6	1.3	1.1
Early light induced protein[a]	DT883583	AAO33591	3e-35	1.1	2.4	1.3	1.3	1.6	1.6
Potential roles in signal transduction or perception									
ACC oxidase[a]	DN740375	AAP13098	1e-81	2.5	2.4	1.5	1.7	1.4	2.2
Phospholipase[a]	DN740357	T00421	8e-25	2.7	3.8	1.3	1.8	1.7	2.1
Immunophilin	DN740369	AAC49391	9e-55	1.3	1.7	1.0	1.1	1.0	1.2
Potential roles in PCD									
Cysteine protease (MecCP1, AY973616)	DN740364	T12041	3e-96	1.4	2.1	1.3	1.4	1.3	1.0
Class IV chitinase	DN740377	NP_191010	5e-78	1.3	1.4	1.2	1.4	1.6	0.3
Stress response									
Dehydrin	DT883584	AAB00554	2e-16	0.9	1.0	1.3	1.6	1.4	0.9
Heat shock protein 70 cognate[a]	DT883585	BAA34919	5e-58	1.6	1.5	1.1	1.1	1.1	1.7
Roles in ion water or metabolite transport									
PIP1 type aquaporin	DN740371	AAF65846	3e-65	1.3	2.4	1.2	1.3	1.7	1.3
PIP2 type aquaporin	DN740353	AAC17529	1e-65	1.4	2.6	1.4	1.3	1.4	1.4
Gamma adaptin	DN740350	NP_176215	2e-55	3.4	4.5	1.3	1.9	1.3	1.2
H^+-PPase	DN740360	AB097115	0.0	0.7	1.0	1.0	1.6	1.1	1.5
ATP/ADP translocase precursor	DT883571	S68205	3e-100	1.6	1.2	0.9	1.0	1.0	0.9
Potential roles in cell wall metabolism and remodelling									
Xyloglucan endotransglycosylase[a]	DT883569	AAD08949	9e-30	1.5	1.4	1.2	1.2	1.1	1.7
UDP-glucose dehydrogenase[a]	DT883565	AAT40106	6e-144	1.9	2.4	1.1	1.5	1.5	1.7
Hydroxyproline-rich glycoprotein	DT883566	AF239615	1e-98	1.4	1.6	1.1	1.1	1.2	1.1
Hydroxyproline-rich glycoprotein	DT883567	NP_180907	3e-25	1.2	1.8	1.1	1.0	1.2	1.4
Extensin	DT883568	S14983	7e-6	1.3	1.8	1.4	1.7	1.4	0.8
Germin-like protein (MecGLP, AY973617)	DN740379	AAM76226	6e-7	1.1	1.1	1.1	1.3	1.2	0.7
Biosynthesis and metabolism									
Cytochrome P_{450} CYP79D1[a]	DN740355	AAF27289	e-121	2.7	4.6	1.3	1.6	1.4	2.8
Cytochrome P_{450} CYP79D2[a]	DN740359	AAF27290	3e-59	2.2	2.3	1.1	1.4	1.2	2.1
Cytochrome P_{450} CYP71E[a]	DN740365	NP_191010	5e-78	3.0	3.9	1.6	1.9	1.5	2.9
Cytochrome P_{450}	DN740376	CAC24711	1e-60	1.4	1.9	1.3	1.6	1.4	1.1
Cytochrome b_5	DT883570	AAM28288	7e-27	2.1	1.9	1.2	1.2	1.3	1.7
UDP glycosyltransferase	DN740366	NP_173653	2e-53	1.9	2.3	1.4	1.3	1.3	1.5
UDP-glycosyltransferase	DT883564	AAR06913	1e-51	1.7	2.3	1.2	1.2	1.1	1.5
L-asparaginase	DT883572	BAC66615	2e-81	1.6	2.2	1.1	1.2	1.3	1.0
Ketol acid reductoisomerase[a]	DT883574	AAU44107	1e-32	1.6	2.0	1.0	1.2	1.0	1.8
Transaldolase protein	DT883573	T07790	6e-96	1.2	1.7	1.2	1.3	1.2	0.9
Arginine decarboxylase	DT883575	BAD97829	1e-76	1.6	1.7	1.0	1.0	1.0	1.0
Sulphite reductase precursor[a]	DT883576	AAG59996	5e-114	1.7	2.8	0.8	1.4	1.3	1.6
Roles in transcription or translation									
Ribosomal protein L5[a]	DT883586	CAA55090	6e-84	1.8	1.7	1.1	1.2	1.0	1.9
Ribosomal protein S10[a]	DT883587	AAM67000	7e-44	1.5	1.9	1.0	1.4	1.1	1.8
Ribosomal protein S16	DT883588	AAF34799	3e-45	1.3	1.7	1.0	1.3	1.1	1.7
Ribosomal protein S29[a]	DN740368	AAP68880	2e-28	1.7	2.0	1.1	1.3	1.4	1.7
Eukaryotic initiation factor eIF4[a]	DT883592	AAN17444	2e-67	1.3	1.9	1.1	1.4	1.3	1.4
ATP-dependent RNA helicase[a]	DT883589	NP_910812	5e-25	No data	1.9	0.8	0.9	1.2	2.0

Table 1 continued

Putative identity	EST accession no.	BLAST hit	*E*-value	Average ratio					
				0 vs. 12 h	0 vs. 24 h	0 vs. 48 h	0 vs. 72 h	0 vs. 96 h	R24 vs. L24
Seryl tRNA synthase	DN740372	O81983	3e-77	1.8	2.1	1.1	1.3	1.2	1.0
Elongation factor eF1a[a]	DN740374	O49169	3e-74	1.2	1.8	1.0	1.4	1.1	2.6
Oligouridylate-binding protein	DN740356	CAB75429	5e-61	1.3	2.0	0.8	1.3	0.9	1.0
Alternative splicing factor SF2a	DT883591	AAF02881	1e-19	1.4	1.4	1.5	1.3	1.1	0.9
Pentatricopeptide repeat (PPR) protein	DT883590	NP_176447	8e-46	2.6	2.0	1.0	1.2	1.1	1.7
Role unknown or uncharacterised									
Putative endopeptidase	DT883593	AAN15475	3e-56	1.3	1.7	1.1	1.0	1.0	1.2
MtN19	DN740354	BAD01246	2e-66	1.4	2.1	1.3	1.4	1.5	1.1
Hypothetical protein	DT883601	AAD10646	1e-34	1.7	1.8	1.3	1.4	1.2	1.3
Putative protein	DT883600	CAB86629	2e-15	1.7	1.6	1.2	1.3	1.4	1.3
Unknown protein	DT883596	XP_469336	3e-20	1.3	1.7	1.2	1.2	1.3	1.2
Unknown protein	DT883597	AAM65782	5e-92	1.6	1.7	1.1	1.2	1.1	1.2
Unknown protein[a]	DN740370	NP_191010	5e-78	1.4	1.9	0.9	1.0	0.9	2.1
No protein match	DT883594	na	na	1.3	1.8	1.1	1.3	1.2	1.0
No protein match	DT883595	na	na	1.7	2.1	1.4	1.4	1.6	1.2
No protein match	DT883598	na	na	1.7	1.8	1.1	1.3	1.3	1.3
No protein match	DT883599	na	na	1.6	1.8	1.1	1.1	1.0	1.2
PPD down-regulated genes									
Cystatin-like protein[a]	DT883603	AAG38521	2e-25	0.4	0.3	0.5	0.4	0.4	1.5
Late embryogenesis-abundant protein LEA5	DT883604	CAA86851	3e-24	0.4	0.4	0.5	0.5	0.4	1.6
Translationally controlled tumour protein[a]	DT883602	AAD10032	6e-77	0.6	0.4	0.7	0.8	0.9	1.3
Auxin repressed protein ARP1	DN740380	AAK25768	6e-20	0.6	0.6	0.6	0.7	0.7	1.2
Auxin repressed protein ARP2	DN740381	AAK25768	7e-33	0.4	0.3	0.5	0.5	0.5	1.1
PWWP domain protein[a]	DN740382	NP_191866	2e-09	0.5	0.4	0.6	0.6	0.5	1.2
Expressed protein	DN740384	NP_568985	1e-8	0.5	0.4	0.5	0.4	0.5	0.7
Expressed protein	DN740383	AAD29798	1e-22	0.4	0.4	0.6	0.6	0.5	1.4
Unknown protein[a]	DN740385	AAM65154	1e-23	0.6	0.4	0.4	0.4	0.4	1.6

The ESTS were selected based on a statistical analysis of their differential expression at various points over the PPD time course and their assigned identities and/or functions were based on BLAST scores (*E* values). Ratio values shown are expression ratios thus a ratio of 2.0 indicates twofold up-regulation whilst a value of 0.5 indicates twofold down-regulation

[a] Genes are identified via their root 24-h post-harvest vs. leaf 24-h post-wounding (R24 vs. L24) analyses as storage-root-wounding-specific and thus potentially PPD-specific. Clones that were fully sequenced and were used in Northern hybridisations have their accession numbers in brackets

during PPD and during the hypersensitive response (HR) of cassava leaves to the incompatible pathovar *Pseudomonas syringae* (Kemp *et al.* 2005; Reilly *et al.* 2001, 2003). EST DN740367 encodes a novel cassava root secretory peroxidase with similarity to cationic plant peroxidases and a predicted pI of 8.53. Since secretory peroxidases play a role in the vascular streaking reactions of PPD this clone was fully sequenced and submitted to the NCBI database (MecPX3, AY973612). Our data also indicate up-regulation of both a thioredoxin peroxidase (DT883578) and a thioredoxin-like protein (DT883579) during PPD. The thioredoxin peroxidases or peroxiredoxins (EC 1.11.1.15) catalyse the reduction of either H_2O_2 or various alkyl hydroperoxides to water and the corresponding alcohol in the presence of thioredoxin proteins. EST DT883580 encodes a glutathione-S-transferase (GST); the GSTs catalyse the conjugation of glutathione to a range of electrophilic, hydrophobic and cytotoxic substrates, thereby reducing their toxicity. During oxidative stress they detoxify metabolites resulting from oxidative damage such as lipid peroxidation and oxidative DNA degradation products (Rentel and Knight 2004). ESTs DT883581 and DT883582 encode metallothionein and quinine oxidoreductase sequences, respectively. The metallothioneins are small Cys-rich proteins involved in ROS scavenging and metal homeostasis (Wong *et al.* 2004). The quinone oxidoreductases serve to break down quinones and semiquinone intermediates resulting from the reduction of quinones. Semiquinones in particular can donate electrons to oxygen, causing the production of superoxide anions (Matvienko *et al.* 2001). ESTs DN740363 and DT883583 show similarity

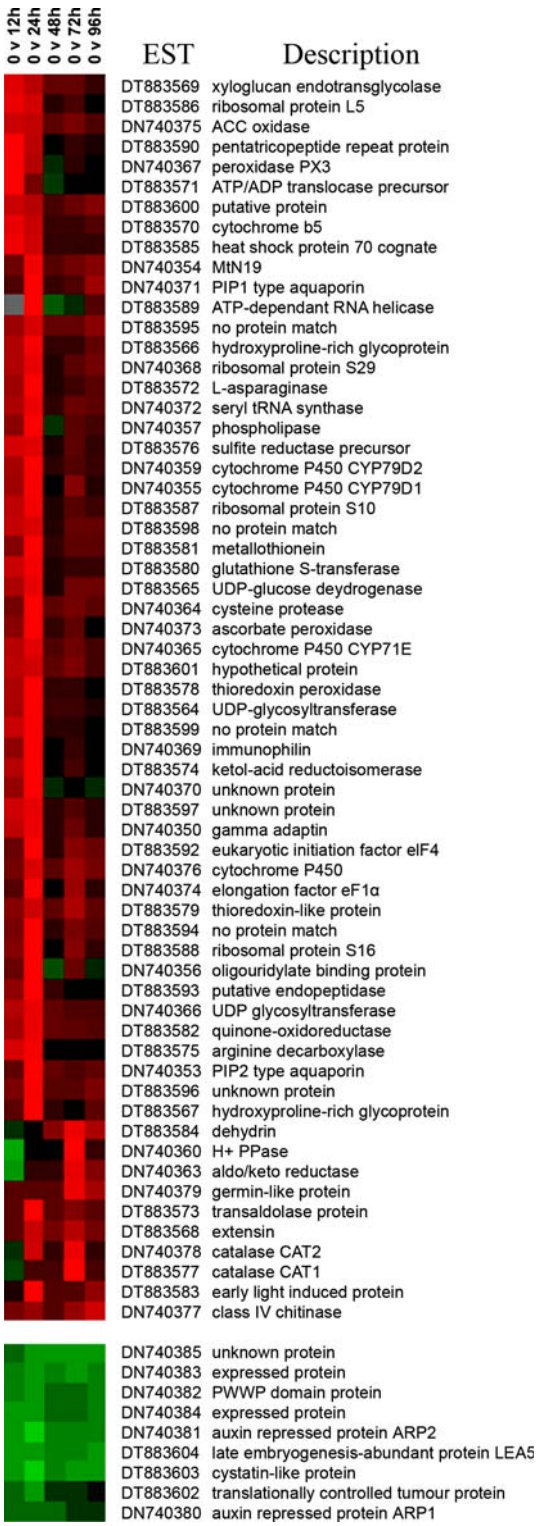

Fig. 2 Heat map showing the expression profile of 72 non-redundant expressed sequence tags showing differential expression over a PPD time course. *Red* indicates up-regulation, *green* indicates down-regulation

to plant auxin-induced aldo/keto reductase (AKR) and to early light inducible protein (ELIP), respectively. Both AKR and ELIP play a role in the detoxification of ROS produced during plant stress (Zhang *et al.* 2005).

Signal transduction and perception

At least three clones identified in this analysis could potentially function in signal transduction or perception events during PPD. ACC oxidase (EC 1.14.17.4) catalyses the last step of ethylene biosynthesis. The plant hormone ethylene is produced in the cassava root within hours after injury (Hirose 1986) and an ACC oxidase sequence (EST DN740375, full length cDNA, MecACCO2, AY973618) with peak expression in the 0 h vs. 12 h microarray hybridisation was identified here. Previous work had identified two other ACC oxidases in cassava, MecACCO1 (EF035079) and MegACCO3 (EF055260), and it is interesting that neither of these members of the gene family were expressed significantly during PPD so as to be identified by the methods used here. ACC oxidase up-regulation in response to wounding has been reported in several plant systems as has differential regulation of ACC oxidase family members (Coupe *et al.* 2003). EST DN740357 encodes a novel cassava phospholipase. In other plants phospholipases are activated in response to cellular and environmental cues and play a role in signal transduction during stress responses via the formation of lipid-derived messengers, as well as in membrane remodelling and degradation (Wang *et al.* 2002). EST DN740369 encodes an immunophilin (FK binding-type peptide isomerase). The function of plant FK-binding proteins (FKBPs) is not yet clear; however, they are proposed to be involved in trafficking of signal proteins in plants. Several plant FKBPs are induced by heat and other stress (Krishna and Kanelakis 2003).

Programmed cell death

Previous studies both in this laboratory and elsewhere (Huang *et al.* 2001) demonstrated up-regulation of genes potentially involved in PCD during PPD. Two novel genes which could potentially function in PCD responses—a cysteine protease (EST DN740364) and a class IV chitinase (EST DN740377) were identified as up-regulated genes in this study. The cysteine proteases are proteolytic enzymes involved in the hydrolysis of proteins and there is increasing evidence that specific proteases may also act as mediators of signal transduction and/or effectors of PCD. They have been implicated in senescence and in response to stresses, such as drought, cold, wounding, ethylene treatment and glucose starvation, which can also induce senes-

cence. PCD may be modulated via the opposing activities of proteases and their cognate inhibitors (Coupe *et al.* 2003; Alesandrini *et al.* 2003). The chitinases (EC 3.2.1.14) catalyse hydrolysis of chitin, a major component of fungal cell walls as well as insect exoskeleton. Plant chitinases were originally classified as pathogenesis-related proteins. More recently a role during PCD has been proposed for class IV chitinases in *B. napus* and *Arabidopsis*. (Kasprzewska 2003; Lima *et al.* 2002).

Stress response

Expressed sequence tags DT883584 and DT883585 encode a dehydrin and an Hsp70 sequence, respectively. The dehydrins are classified as group II late embryogenesis abundant (LEA) proteins. They are hydrophilic, thermostable and contain a significant proportion of charged amino acids, implying that they may function as structure stabilisers with detergent- and chaperone-like properties. They are expressed during periods of water stress, or in response to other environmental stresses where osmotic stress is a component of the stress mechanism (Heyen *et al.* 2002). Plant Hsp70 genes are induced by a variety of stress conditions including heat shock, wounding, water deficit, ABA and cold, and could play a general role in stress adaptation (Li *et al.* 1994). The water loss during PPD probably imposes water-deficit and osmotic stresses on the root, which would account for the expression of these two genes.

Ion, water or metabolite transport

The ESTs DN740371 and DN740353 encode plasma-membrane intrinsic proteins (PIP), PIP1 and PIP2 type aquaporins, respectively. The aquaporins are membrane channel proteins that regulate water permeability by increasing water permeation across biological membranes. They are members of the major intrinsic protein family. Since stress may alter water regulation, aquaporins have been proposed to be involved in plant stress responses (Baiges *et al.* 2002). EST DN740350 encodes a gamma adaptin. The adaptins are subunits of adaptor protein complexes, which are involved in intracellular vesicle transport (Lam *et al.* 2002). EST DN740360 encodes a proton translocating inorganic pyrophosphatase (H^+-PPase) (also called V type PPase), a proton pump found in most land plants and in some algae, protozoa, bacteria and archaea. It is primarily localised to the vacuolar membrane of plant cells and hydrolyses pyrophosphate, a by-product of metabolic processes such as protein, starch and cellulose synthesis, coupled to active proton transport across the vacuolar membrane and leading to acidification of the vacuole. Changes in H^+-PPase activity occur in response to stress including nutrient stress, anoxia and chilling (Etienne *et al.* 2002). EST DT883571 encodes an ATP/ADP translocase, an anti-porter of the inner plastid and mitochondrial membrane. ATP is usually exported to the cytoplasm and ADP imported for further ATP synthesis.

Cell wall remodelling and repair

Current models of cell wall structure postulate a pectin matrix, in which is embedded a network of cellulose microfibrills crosslinked to xyloglucans, and a network of structural proteins crosslinked together and to other wall components. Important protein families involved in cell wall metabolism include enzymes for monosaccharide activation and interconversion, polysaccharide synthesis, reassembly and degradation enzymes, structural proteins and glycoprotein glycosyltransferases (Girke *et al.* 2004). EST DT883569 identified in Table 1 encodes a xyloglucan endotransglycosylase (XET) that shows rapid up-regulation after harvest. The XETs (EC 2.4.1.207) are reassembly and degradation enzymes that function to break and join xyloglucan polymers, the major hemicellulose of the primary cell wall, thereby reinforcing the wall during growth and in response to mechanical strain (Antosiewicz *et al.* 1997). EST DT883565 encodes a uridine diphosphate (UDP)-glucose dehydrogenase (EC1.1.1.22), an enzyme controlling the biosynthesis of sugar nucleotides required for biosynthesis of hemicellulose components (Tenhaken and Thulke 1996). HRGP/extensin transcripts commonly show up-regulation in response to wounding, pathogen infection, ethylene treatment, and during development. Once synthesised, extensin monomers rapidly become insolubilised in the cell wall in a reaction involving H_2O_2 and mediated by a cell wall peroxidase (Showalter 1993), thereby sealing and strengthening the cell wall. EST DT883566 described here was identical to a previously described cassava HRGP, cMeHRGP1 (AF239615), which showed up-regulation in cassava cell-suspension cultures treated with glucan cell wall elicitor, and in injured cassava storage roots (Gómez-Vásquez 2000; Han *et al.* 2001), while EST DT883567 encodes a novel cassava HRGP. Transcript DN740379 encodes a germin-like protein (GLP). The germins and GLPs are a large and diverse class of plant cell wall proteins belonging to the cupin superfamily. They are implicated in cell wall remodelling

during both development and stress responses, although the exact nature of their role in these processes is unclear (Mathieu *et al.* 2003).

Biosynthesis and metabolism

Previous data had indicated that PPD is an active process involving the synthesis of novel compounds as well as active protein synthesis. As might be expected, a number of up-regulated genes involved in biosynthesis and metabolism, and in transcription or translation were identified in this analysis. Five discrete cytochrome sequences showed up-regulation during PPD, a cytochrome b_5 gene and four cytochrome P_{450} encoding genes. The cytochrome P_{450} proteins are one of the largest families of proteins in plants with 272 (including 26 pseudogenes) in *Arabidopsis*. They catalyse a diverse range of reactions, generally based on activation of molecular oxygen with insertion of one its atoms into the substrate and reduction of the other to produce H_2O and are classified as mono-oxygenases. The substrates and roles of plant cytochrome P_{450} s are diverse and include synthesis of structural molecules such as lignin and suberin, synthesis of hormones and signalling molecules, pigments, antioxidants and defence molecules, catabolism of signalling molecules such as abscisic acid (ABA), and detoxification of exogenous molecules such as pesticides and pollutants (Werck-Reichhart *et al.* 2002). Three of the sequences described here correspond to previously described cassava cytochrome P_{450} genes. ESTs DN740355 and DN740359 encode cassava CYP79D1 and CYP79D2 cytochrome P_{450}s, respectively, and are involved in the synthesis of the cyanogenic glycosides linamarin and lotaustralin in the root (Andersen *et al.* 2000). EST DN740365 was identical to cassava cytochrome CYP71E (c15, AY217351). The transcript was previously identified by differential screening to identify cassava storage-root-specific promoters, and was expressed in storage roots but not leaves. Sequencing and analysis of the cognate promoter identified a number of *cis*-acting regulatory motifs including a wound-responsive motif, an ethylene response element, an ABA-responsive element, and two methyl jasmonate response elements (Zhang *et al.* 2003). EST DN740376 encodes a novel cassava cytochrome P_{450}, whilst EST DT883570 encodes a novel cassava cytochrome b_5. In yeast and mammalian cells cytochrome b_5 acts as a redox intermediate in biosynthetic reactions. In plants it is proposed to act as a direct or indirect electron donor in the biosynthesis of products such as sterols, terpenoids and gibberellic acids (Martsinkovskaya *et al.* 1999). ESTs DN740366 and DT883564 encode two discrete UDP glycosyltransferases. The glycosyltransferases (EC 2.4.x.y) are responsible for the biosynthesis of disaccharides, oligosaccharides and polysaccharides and catalyse the formation of glycosidic bonds. Such glycosylation reactions often represent the end point of higher plant secondary metabolite synthesis and, in addition, many classes of plant hormone exist in a glycosylated form including auxins, gibberellins, cytokinins and ABA (Lim *et al.* 2003). The sequences presented here showed no significant sequence similarity to previously isolated cassava glycosyltransferases available on the NCBI database (Hughes and Hughes 1994). EST DT883572 encodes the enzyme L-asparaginase (EC 3.5.1.1), a key enzyme involved in Asn hydrolysis in plants. Ammonia is released in the reaction and is utilised for the synthesis of nitrogen containing compounds, including amino acids required for protein synthesis (Casado *et al.* 1995). EST DT883574 encodes a ketol-acid reductoisomerase (EC 1.1.1.86), an enzyme involved in synthesis of branched chain amino acids including isoleucine and valine (Dumas *et al.* 1993). EST DT883573 encodes transaldolase (EC 2.2.1.2), an enzyme of the non-oxidative branch of the plant pentose phosphate pathway that is involved in transfer of carbohydrates from primary to secondary metabolism allowing synthesis of aromatic amino acids, flavonoids and lignin (Caillau and Quick 2005). EST DT883575 encodes an arginine decarboxylase (ADC, EC 4.1.1.19), involved in the synthesis of putrescine. Transient increases in ADC activity and putrescine levels have been described in a number of plant stress and wounding responses (Perez-Amador *et al.* 2002). EST

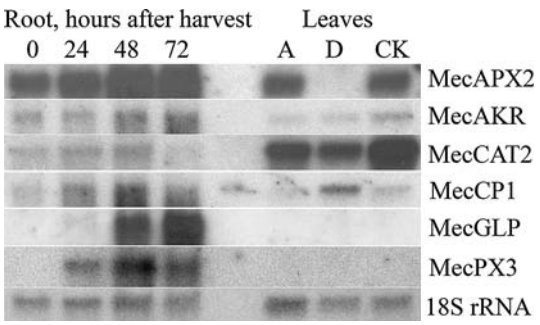

Fig. 3 Expression profiles of selected genes. Northern blots of cassava mRNA to ascertain the expression profiles of the genes corresponding to selected cDNA probes over a PPD time course (time in hours) and in leaves (*A* wounded but attached leaves, *D* wounded and detached leaves, *CK* control, un-wounded attached leaves)

883576 encodes sulphite reductase (EC 1.8.7.1) a key enzyme of sulphate assimilation in plants (Bork *et al.* 1998).

Transcription and translation

Eight of the up-regulated clones described in Table 1 code for elements of the protein translation machinery: ribosomal proteins L5, S10, S16 and S29; eukaryotic initiation factor eIF4 involved in mRNA recruitment; an ATP-dependent RNA-helicase involved in unwinding of mRNA secondary structure; a seryl tRNA synthase involved in definition of codon identity; and an elongation factor eF1α involved in binding of aminoacyl tRNA to ribosomes during the elongation step of translation. EST DN740356 encodes an oligouridylate binding protein (UBP) with highest similarity to *Nicotiana plumbaginifolia* UBP1. UBP1 enhances accumulation of certain classes of transcript via enhanced mRNA stability (Lambermon *et al.* 2000). EST DT883591 encodes an alternative splicing factor SF2a. Alternative splicing profiles are affected by environmental stress conditions, and are thought to be mediated both by transcriptional regulation of splicing factors and alternative splicing of the splicing factors themselves (Iida *et al.* 2004). EST DT883590 encodes a pentatricopeptide repeat (PPR) protein. PPR proteins contain a characteristic 35 amino acid motif repeated in a tandem array. Most plant PPR proteins are targeted to mitochondria or chloroplasts and are thought to be involved in RNA processing or translation (Kazama and Toriyama 2003).

Confirmation of expression profiles of selected up-regulated clones

To validate and extend the microarray data a number of cDNA clones corresponding to up-regulated ESTs from the microarray analysis were fully sequenced and used as probes for Northern blotting experiments (Fig. 3). Fully sequenced clones include the cognate cDNAs to ESTs for ascorbate peroxidase MecAPX2 (AY973722), a putative auxin-induced protein aldo/keto reductase MecAKR (AY973615), a catalase MecCAT2 (AY973614), a cysteine protease MecCP1 (AY973616), a germin-like protein MecGLP (AY973617) and a secretory peroxidase MecPX3 (AY973612). Northern blot data confirmed that the expression patterns of the corresponding genes were up-regulated during a PPD time course. The precise timing of peak expression observed was not identical to the microarray data (Table 1; Fig. 2). However, it had previously been observed that the symptoms of PPD initiate and progress more rapidly under field conditions in Colombia than under laboratory conditions at Bath, and this may account for earlier observed peaks of expression in the microarray data, which used targets derived from mRNA isolated from field-grown roots. The observed expression profiles in different cassava tissues indicates that at least some of the genes are potentially PPD-specific, i.e. expressed only during root deterioration and not in un-wounded root; or wounded or un-wounded leaves. Of particular interest were the germin-like protein (MecGLP) and the secretory peroxidase (MecPX3), which showed putative PPD-specificity. The latter was induced to high levels within the 24 first hours of harvest according to both microarray and Northern blotting data. The ascorbate peroxidase (MecAPX2) showed contrasting expression patterns in wounded leaves, being expressed when the wounded leaf remained attached to the plant but not when it was detached. The cysteine protease (MecCP1) showed the opposite expression pattern in leaves, with up-regulation in wounded detached leaves, but background expression in wounded attached leaves. The nucleotide sequence of MecCPI had 80% identity with BOCP3 – a senescence associated cysteine proteases in broccoli florets (Coupe *et al.* 2003). Injured cassava roots which remain attached to the plant are capable of normal wound healing (Mwenje *et al.* 1998), thus it is tempting to speculate that severance of the root or leaf from the plant triggers components of a senescence or PCD pathway.

PPD down-regulated genes

Sequences identified as showing down-regulation in the storage root during PPD include a cystatin like protein (EST DT883603), a LEA protein (EST DT883604), a translationally controlled tumour protein (TCTP) (DT883604), two clones with homology to auxin repressed proteins (ARP) designated ARP1 and ARP2 (DN740380 and DN740381), and a PWWP domain protein (DN740380).

Plant cysteine protease inhibitors or cystatins regulate endogenous cysteine protease activity during various biological processes including defence, response to biotic and abiotic stress, embryogenesis and seed germination, and in PCD (Shyu *et al.* 2004). Generation of ROS plays a key role in PCD in both plant and animal cells; at low levels antioxidant enzymes are induced, though when ROS levels reach a critical threshold a PCD pathway is triggered. For example, in cultured soybean cells PCD-inducing oxidative stress triggered

expression of a set of cysteine proteases, while ectopic expression of an endogenous cystatin inhibited cysteine protease activity and blocked PCD (Solomon *et al.* 1999).

The LEA proteins are a group of loosely related proteins that are involved in desiccation tolerance in plants. Although their precise mode of action remains unclear proposed mechanisms include water retention, ion sequestration, direct protection of proteins or membranes and renaturation of unfolded proteins. The down-regulated EST DT883604 described here showed homology to LEA5/D73 type LEAs. These have recently been reclassified as class V LEAs (Wise 2003).

Translationally controlled tumour proteins are tubulin-binding proteins that associate with microtubules in a cell-cycle-dependant manner and play a role in cell growth-related activities. They have been primarily studied in mammalian systems and their precise role in plants remains unclear. In pea a TCTP (PsCaP23) and a 60S ribosomal protein L41 were up-regulated within 15 min of root cap induction, where their expression was localised to dividing cells within the root cap, suggesting that plant TCTPs, like their mammalian counterparts, are involved in cell proliferation (Woo and Hawes 1997). In humans a TCTP is involved in the modulation of translation elongation, via interaction with the elongation factors eEF1α and eEF1β. Decreased TCTP expression levels promote apoptosis, and strong down-regulation has been observed during tumour reversion/suppression (Cans *et al.* 2003).

The ARP gene family is specific to higher plants, and members of the family contain highly conserved N-terminal and C-terminal domains. The plant hormone auxin is involved in the regulation of a range of growth and developmental processes; however, to date little is known about the precise role of ARP genes. An ARP homologue from *Capsicum annuum* was induced in *X. campestris* and *P. capsici* infected tissues but was not induced in response to wounding, salicylic acid, jasmonic acid or ethephon treatment (Jung and Hwang 2000). The *Robinia pseudoacacia* RpARP gene was down-regulated by auxin, and such down-regulation was dependant on the presence of metabolisable sugar and on protein synthesis. Deletion constructs of RpARP with or without untranslated regions (UTRs) indicated that the protein was additionally regulated post-transcriptionally by auxin and this is mediated via the UTRs. Although a number of mRNA instability motifs have been identified in the UTRs of other genes, no known motif was found in RpARP (Park and Han 2003). Intriguingly, although the available coding regions of cassava ARP1 and ARP2 are identical, the full sequences of the cognate cDNAs reveal a difference in their 3′ UTRs with a ~40bp insertion in ARP1 (AY973620) relative to ARP2 (AY973621).

EST DN740380 showed highest similarity to an *Arabidopsis* PWWP domain protein. The PWWP domain is a conserved protein domain based on a central core ''proline–tryptophan–tryptophan–proline'', hence its designation (pfam PF00855). The domain is located at either the N- or C-terminus of the protein and is found in proteins involved in cell division, growth and differentiation. Where data are available, PWWP proteins appear to be nuclear proteins and are often DNA binding proteins acting as transcription factors. It is proposed that the domain acts as a site for protein–protein interaction and may modulate transcription via chromatin remodelling (Stec *et al.* 2000).

Microarray analysis to identify PPD-specific and storage-root-specific genes

In addition to genes that were up- or down-regulated during PPD, the microarray hybridisations and analyses were capable of identifying genes whose expression change significantly (up or down) during PPD (storage root wounding) but not during leaf wounding, and genes expressed in storage roots but not in leaves (un-wounded or wounded). While not categorical definitions, the former can be considered as PPD-specific, or perhaps more accurately storage-root-wounding-specific, and the latter root-specific. Twenty four storage-root-wounding-specific genes were identified and are indicated in Table 1. They were distributed between the all the several functional classes of up- and down-regulated PPD genes discussed above (Table 1). Surprisingly, while this storage-root-wounding-specific group included the secretory peroxidase (MecPX3), also identified by Northern hybridisation (Fig. 3) and discussed above, the germin-like protein (MecGLP) did not fulfil the microarray criteria.

Of the group of 21 storage root-specific genes, none were common to the up-regulated PPD genes, though four were identical to the smaller group of down-regulated genes: cystatin-like protein (DT883603), TCTP (DT883602), ARP1 (DN740380) and an unidentified expressed protein (DN740383) (Table 2; Fig. 4). Interestingly, the first two were also common to the PPD-specific group. The remaining 17 genes in this root-specific group were novel and fell into several functional groups (Table 2). Of these, EC591287 was very highly similar to an allergenic-related protein from cassava (AY101376, de Souza *et al.* 2004) and also

Table 2 Root-specific ESTs

Putative identity	EST accession no.	BLAST hit	*E*-value	Average ratio		
				R0 vs. L0	R0 vs. L24	R24 vs. L24
Potential roles in signal transduction or perception						
Auxin-repressed protein-like protein ARP1	DN740380	AAX84677	1e-50	1.67	1.65	0.64
GTP-binding protein	EC591276	AAM12880	2e-107	1.11	0.80	0.64
Nuclear transport factor, putative	EC591277	AAM63803	1e-55	1.53	1.53	0.97
Potential roles in cell wall metabolism and remodelling						
Cell wall-plasma membrane linker protein	EC591278	CAA64425	5e-30	1.04	0.99	0.40
Roles in transcription or translation						
Translation initiation factor	EC591279	NP_175831	8e-54	1.30	1.35	0.95
Putative ribosomal protein L10a	EC591280	BAD28853	2e-82	1.19	1.24	0.63
Structural constituent of ribosome	EC591281	NP_191670	4e-44	1.27	1.12	0.64
Nucleic acid binding	EC591282	NP_568522	7e-42	1.07	1.13	0.91
Roles in protein modification						
Electron transporter/thiol-disulphide exchange	EC591275	NP_187483	2e-39	1.76	1.74	1.44
Polyubiquitin	EC591283	AAB68045	3e-79	1.22	0.97	0.49
Role unknown or uncharacterised						
Cystatin-like protein	DT883603	AAG38521	4e-25	1.85	1.56	0.60
Translationally controlled tumour protein	DT883602	AAD10032	1e-58	1.28	0.90	0.40
M. esculenta allergenic-related protein Pt2L4I	EC591287	AY101376	0.0	1.73	1.89	1.45
Arabidopsis expressed	DN740383	AAD29798	1e-22	2.09	1.73	1.04
Unknown protein	EC591291	AAL87368	1e-39	1.45	1.15	0.83
Conserved hypothetical protein	EC591292	ABE84267	2e-35	1.22	1.51	0.72
Unknown protein	EC591284	ABA81870	1e-18	2.04	2.02	1.09
Unknown protein	EC591285	NP_565499	1e-20	2.08	1.54	0.92
Unknown protein	EC591286	NP_568985	5e-30	0.69	0.62	0.48
No protein match	EC591289	na	na	1.61	1.62	1.29
No protein match	EC591290	na	na	1.52	1.11	0.72

Root-specific ESTs were selected on their relative expression patterns using root and leaf un-wounded and wounded targets. Assigned identities were based on BLAST scores (*E* values)

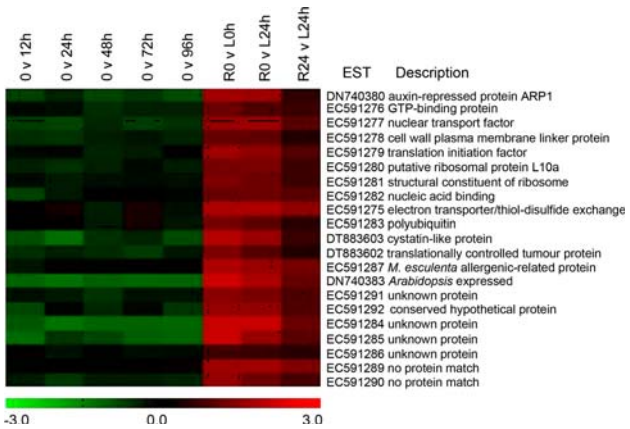

Fig. 4 Heat map showing the expression profiles of 21 non-redundant expressed sequence tags showing differential expression in deteriorating cassava storage roots and leaves. *Red* indicates up-regulation, *green* indicates down-regulation

bears high similarity to another cassava gene for a glutamic acid-rich protein (AY217354) whose promoter shows strong expression in vascular tissues and during the secondary growth of cassava storage roots (Zhang *et al*. 2003).

Discussion

The approach employed here has led to the identification of 73 genes whose expression changed significantly during PPD. In addition, a comparison between harvested storage roots and un-wounded and wounded leaves led to the identification of a 24 member subset of these genes as being storage-root-wounding-specific and another 21 genes, 4 of which were included in the PPD down-regulated group, as storage-root-specific. It is reassuring that many of the genes studied previously as ''likely candidates'' involved in PPD have also emerged in this more rigorous and ''operator neutral'' microarray approach (Han *et al*. 2001; Reilly *et al*. 2001, 2003).

A recent examination of the cassava fibrous and storage root proteome led to the identification of 237 protein spots of which 104 were listed (Sheffield *et al*. 2006). The classification of these proteins into functional groups placed them in many of the classes identified in this study. However, only six proteins, ascorbate peroxidase, catalase CAT1, GST, EF1α, transaldolase and TCTP, were common to both. The EF1α was identified here as up-regulated during PPD and storage-root-wounding-specific, while the TCTP

was down-regulated, storage-root-wounding-specific and root-specific.

Huang *et al*. (2001) used a cDNA-ALFP fingerprinting approach to identify transcript-derived fragments (TDFs) of genes whose expression altered during cassava PPD; these included TDFs for genes involved in oxygen scavenging and stress responses. To our knowledge their TDF sequences are not available on public databases, thereby preventing direct sequence comparisons. However, a peroxidase, glycosyltransferase, elongation factor EF1α, heatshock protein 70, three ribosomal proteins, and two cytochrome P_{450} sequences were shown to be up-regulated in both Huang *et al*. (2001) and this study. While no comparable microarray study of PPD exists, that of Lopez *et al*. (2005), using a microarray of cassava ESTs, enriched for starch biosynthetic and bacterial blight-responsive sequences, to dissect cassava's response to the pathogen *X. axonopodis*, merits comparative examination. Both studies identified some common functional groups of genes whose expression was up-regulated: ROS modulation, cell wall metabolism, and transcription/translation. For example, both identify several ESTs for ROS-modulating enzymes that fall into essentially the same classes or gene families; however, a close sequence comparison shows that only one pair of ESTs for ascorbate peroxidase (DN740373 and CK649432), were essentially identical with respect to the regions sequenced (data not shown). Other common genes include cytochrome P_{450}s, of which DN740367 and CK63363 are identical (data not shown), and aquaporins. These common genes confirm that, while the biotic and abiotic stress responses analysed in these two studies are strikingly different, there are major areas of overlap between plant stress responses in general.

The characterisation of genes expressed during PPD and isolated through random and directed cloning approaches had led to the generation of a model that placed ROS and their modulation centre-stage in the PPD response, supported by enzymes involved in PCD and more general defence responses (Reilly *et al*. 2003). The data presented in this paper, which have been collected by a more thorough and statistically robust approach, provide further support for that model. The ROS produced and triggered by the wounding associated with cassava root harvesting probably fulfil multiple roles: as initial signals that trigger signal transduction pathways that lead to changes in gene expression; as components in cellular development or repair, such as cell wall formation; as oxidants of cellular components and molecules, such as of scopoletin that leads to the vascular streaking of PPD; and cellular damage or PCD. All these ROS-related events were observed during PPD and increased activity of genes involved in several of these processes was detected during this study, confirming an important role for ROS in the development of PPD.

The expression of some genes associated with PCD or apoptosis were significantly altered: cysteine protease (MecCP1, AY973616) and class IV chitinase (DN740377) were up-regulated, whilst the cystatin-like protein (DT883603) and TCTP (DT883602), were down-regulated. MecCP1 was up-regulated in wounded detached leaves but not in wounded attached leaves, suggesting that the former had embarked on a PCD pathway while the latter had not. Cystatins regulate cysteine protease inhibitors, so the up-regulation of the former coupled with the down-regulation of the latter would be expected to enhance the hydrolysis of proteins and thereby the PCD response of the root. TCTPs are highly conserved proteins that appear to be common to all eukaryotes; they are important in cell cycle progression and have anti-apoptotic activity. While TCTP is not tumour-specific, its expression increases during mammalian tumour formation and its down-regulation is associated with reversion of cells to a normal phenotype (Bommer and Thiele 2004). The function of TCTP has not been studied in plants; however, its down-expression observed during PPD is consistent with the anti-apoptotic role observed in animal systems. Certainly, the down-regulation of two anti-apoptotic genes and the parallel up-regulation of two PCD genes is consistent with a role for PCD responses during PPD. Additional circumstantial data supporting this view comes from the observation that the coumarin, scopoletin, that accumulates during PPD and is responsible for much of the fluorescence under UV light (Buschmann *et al*. 2000b), accelerates the PPD response when applied exogenously to the cassava root (Wheatley and Schwabe 1985). Recently it has been reported that scopoletin has an inhibitory effect and induces apoptosis in human cancer cell lines (Kim *et al*. 2005; Adams *et al*. 2006). These reports raise the intriguing question as to whether scopoletin may also have a pro-apoptotic role in cassava PPD.

These data are not sufficient to determine whether in the harvested cassava storage root the accumulation of high levels of ROS is a symptom or component of PCD, or vice versa. It is possible that the rapid oxidative burst detected within 15 min of harvest (Reilly *et al*. 2003) generates the initial signals that trigger PCD of which high levels of damaging ROS are a major component. Nonetheless, the results presented

here provide knowledge and tools with which to approach answering these questions through modulating the root's anti-oxidant or anti-PCD status via transgenes driven by specific promoters.

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References

Adams M, Efferth T, Bauer R (2006) Activity-guided isolation of scopoletin and isoscopoletin, the inhibitory active principles towards CCRF-CEM leukaemia cells and multi-drug resistant CEM/ADR5000 cells, from *Artemisia argyi*. Planta Med 72:862–864

Alesandrini F, Mathis R, Van de Sype G, Didier Hérouart D, Puppo A (2003) Possible roles for a cysteine protease and hydrogen peroxide in soybean nodule development and senescence. New Phytol 158:131–138

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Andersen MD, Busk PK, Svendsen I, Moller BL (2000) Cytochromes P-450 from cassava (*Manihot esculenta* Crantz) catalyzing the first steps in the biosynthesis of the cyanogenic glucosides linamarin and lotaustralin. Cloning, functional expression in *Pichia pastoris*, and substrate specificity of the isolated recombinant enzymes. J Biol Chem 275:1966–1975

Antosiewicz D, Purugganan M, Poliensky D, Braam J (1997) Cellular localisation of *Arabidopsis* xyloglucan endotransglycosylase related proteins during development and after wind stimulation. Plant Physiol 115:1319–1328

Baiges I, Schaffner AR, Affenzeller MJ, Mas A (2002) Plant aquaporins. Physiol Plant 115:175–182

Bommer UA, Thiele BJ (2004) The translationally controlled tumour protein (TCTP). Int J Biochem Cell Biol 36:379–385

Booth RH (1976) Storage of fresh cassava (*Manihot esculenta*). Post-harvest deterioration and its control. Exp Agric 12:103–111

Bork C, Schwen J, Hell R (1998) Isolation and characterization of a gene for assimilatory sulfite reductase from *Arabidopsis thaliana*. Gene 212:147–153

Buschmann H, Reilly K, Rodriguez MX, Tohme J, Beeching JR (2000a) Hydrogen peroxide and flavan-3-ols in storage roots of cassava (*Manihot esculenta* Crantz) during postharvest deterioration. J Agric Food Chem 48:5522–5529

Buschmann H, Rodriguez MX, Tohme J, Beeching JR (2000b) Accumulation of hydroxycoumarins during post-harvest deterioration of tuberous roots of cassava (*Manihot esculenta* Crantz). Ann Bot 86:1153–1160

Caillau M, Quick P (2005) New insights into plant transaldolase. Plant J 43:1–16

Cans C, Passer BJ, Shalak V, Nancy-Portebois V, Crible N, Amzallag N, Allanic D, Tufino R, Argentini M, Moras D, Fiucci G, Goud B, Mirande M, Amson R, Telerman A (2003) Translationally controlled tumor protein acts as a guanine nucleotide dissociation inhibitor on the translation elongation factor eEF1A. Proc Natl Acad Sci USA 100:13892–13897

Casado A, Caballero J, Franco A, Cardenas J, Grant M, Munoz-Blanco J (1995) Molecular cloning of the gene encoding L-asparaginase gene of *Arabidopsis thaliana*. Plant Physiol 108:1321–1322

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acid Res 31:3497–3500

Chu G, Narasimhan B, Tibshirani R, Tusher V (2002) SAM "significance analysis of microarrays" users guide and technical document. http://www-stat.stanford.edu/~tibs/SAM/

Coupe SA, Sinclair BK, Watson LM, Heyes JA, Eason JR (2003) Identification of dehydration-responsive cysteine proteases during post-harvest senescence of broccoli florets. J Exp Bot 54:1045–1056

Desikan R, Mackerness SAH, Hancock JT, Neill SJ (2001) Regulation of the *Arabidopsis* transcriptome by oxidative stress. Plant Physiol 127:159–172

de Souza CRB, Carvalho LJCB, Cascardo JCD (2004) Comparative gene expression study to identify genes possibly related to storage root formation in cassava. Protein Pept Lett 11:577–582

Dumas R, Curien G, Derose R, Douce R (1993) Branched chain amino acid synthesis in plants—molecular cloning and characterization of the gene encoding acetohydroxy acid isomeroreductse (ketol-acid reductoisomerase) from *Arabidopsis thaliana*. Biochem J 294:821–828

Eisen M, Spellman P, Botstein D, Brown P (1998) Cluster analysis and display of genome wide expression patterns. Proc Natl Acad Sci USA 95:14863–14868

Etienne C, Moing A, Dirlewanger E, Raymond P, Monet R, Rothan C (2002) Isolation and characterization of six peach cDNAs encoding key proteins in organic acid metabolism and solute accumulation: involvement in regulating peach fruit acidity. Physiol Plant 114:259–270

Girke T, Lauricha J, Tran H, Keegstra K, Raikhel N (2004) The cell wall navigator database. A systems based approach to organism unrestricted mining of protein families involved in cell wall metabolism. Plant Physiol 136:3003–3008

Gómez-Vásquez R (2000) Defence responses in cassava suspension cells treated with elicitors. University of Bath, Ph.D. dissertation, University of Bath, UK

Han Y, Gómez-Vásquez R, Reilly K, Li H, Tohme J, Cooper RM, Beeching JR (2001) Hydroxyproline-rich glycoproteins expressed during stress responses in cassava. Euphytica 120:59–70

Heyen B, Alsheike M, Smith E, Torvik C, Seals D, Randall S (2002) The calcium binding activity of a vacuole associated dehydrin like protein is regulated by phosphorylation. Plant Physiol 130:675–687

Hirose S (1986) Physiological studies on post-harvest deterioration of cassava plants. Jpn Agric Res Q 19:241–252

Hirose S, Data ES, Quevedo MA (1984) Changes in respiration and ethylene production in cassava roots in relation to post-harvest deterioration. In: Uritani I, Reyes ED (eds) Tropical root crops: postharvest physiology and processing. Japan Scientific Societies, Tokyo, pp 83–98

Huang J, Bachem C, Jacobsen E, Visser RGF (2001) Molecular analysis of differentially expressed genes during postharvest deterioration in cassava (*Manihot esculenta* Crantz) tuberous roots. Euphytica 120:85–93

Hughes J, Hughes M (1994) Multiple secondary plant product UDP-glucose glucosytransferase genes expressed in cassava (*Manihot esculenta* Crantz) cotyledons. DNA Seq 5:41–49

Iida K, Seki M, Sakurai T, Satou M, Akiyama K, Toyada T, Konagaya A, Shinoaki K (2004) Genome wide analysis of alternative pre-mRNA splicing in *Arabidopsis thaliana* based on full length cDNA sequences. Nucleic Acids Res 32:5096–5103

Jung HW, Hwang BK (2000) Isolation, partial sequencing, and expression of pathogenesis-related cDNA genes from pepper leaves infected by *Xanthomonas campestris* pv. *vesicatoria*. Mol Plant Microbe Interact 13:136–142

Kasprzewska A (2003) Plant chitinases—regulation and function. Cell Mol Biol Lett 8:809–824

Kazama T, Toriyama K (2003) A pentatricopeptide repeat containing gene that promotes the processing of aberrant *atp6* RNA of cytoplasmin male-sterile rice. FEBS Lett 544:99–102

Kemp B, Beeching JR, Cooper RM (2005) cDNA-AFLP reveals genes differentially expressed during the hypersensitive response of cassava. Mol Plant Pathol 6:113–123

Kim EK, Kwon KB, Shin BC, Seo EA, Lee YR, Kim JS, Park JW, Park BH, Ryu DG (2005) Scopoletin induces apoptosis in human promyeloleukemic cells, accompanied by activations of nuclear factor kappa B and caspase-3. Life Sci 77:824–836

Krishna P, Kanelakis KC (2003) The 70-kDa protein bound to hsp90 in wheat germ lysate is a plant homologue of animal Hop. Physiol Plant 119:456–462

Lalaguna F, Agudo M (1989) Relationship between changes in lipid with ageing of cassava roots and senescence parameters. Phytochem 28:2059–2062

Lam BCH, Sage TL, Bianchi F, Blumwald E (2002) Regulation of ADL6 activity by its associated molecular network. Plant J 31:565–576

Lambermon MH, Simpson GG, Wieczorek Kirk DA, Hemmings-Mieszczak M, Klahre U, Filipowicz W (2000) UBP1, a novel hnRNP-like protein that functions at multiple steps of higher plant nuclear pre-mRNA maturation. EMBO J 19:1638–1649

Li Q, Anderson J, Guy C (1994) A cDNA clone encoding a spinach 70 kilodalton heat shock cognate. Plant Physiol 105:457–458

Lim E, Baldauf S, Li Y, Elias L, Worrall D, Spencer S, Jackson R, Taguchi G, Ross J, Bowles D (2003) Evolution of substrate recognition across a multigene family of glycosyltransferases in *Arabidopsis*. Glycobiology 13:139–145

Lima VM, Magioli C, Gerhardt LBD, Tarre E, Menezes RMG, Sachetto-Martins G, Margis-Pinheiro M (2002) Bean class IV chitinase promoter is modulated during plant development and under abiotic stress. Physiol Plant 116:512–521

Lopez C, Soto M, Restrepo S, Piegu B, Cooke R, Delseny M, Tohme J, Verdier V (2005) Gene expression profile in response to *Xanthomonas axonopodis* pv. *manihotis* infection in cassava using a cDNA microarray. Plant Mol Biol 57:393–410

Martsinkovskaya A, Poghosyan Z, Haralampidis K, Murphy D, Hatzopoulos P (1999) Temporal and spatial gene expression of cytochrome b5 during flower and fruit development in olives. Plant Mol Biol 40:79–90

Mathieu M, Neutelings G, Hawkins S, Grenier E, David H (2003) Cloning of a pine germin-like protein (GLP) gene promoter and analysis of its activity in transgenic tobacco Bright Yellow 2 cells. Physiol Plant 117:425–434

Matvienko M, Wojtowwicz A, Wrobel R, Jamison D, Goldwasser Y, Yoder J (2001) Quinone oxidoreductase message levels are differentially regulated in parasitic and non parasitic plants exposed to allelopathic quinones. Plant J 25:375–387

Mwenje E, Ride JP, Pearce RB (1998) Distribution of Zimbabwean *Armillaria* groups and their pathogenicity on cassava. Plant Pathol 47:623–634

Park S, Han KH (2003) An auxin-repressed gene (RpARP) from black locust (*Robinia pseudoacacia*) is posttranscriptionally regulated and negatively associated with shoot elongation. Tree Physiol 23:815–823

Perez-Amador M, Leon J, Green P, Carbonell J (2002) Induction of the argentine decarboxylase ADC2 gene provides evidence for the involvement of polyamines in the wound response in *Arabidopsis*. Plant Physiol 130:1454–1463

Reilly K, Han Y, Tohme J, Beeching JR (2001) Isolation and characterisation of a cassava catalase expressed during post-harvest physiological deterioration. Biochim Biophys Acta 1518:317–323

Reilly K, Gómez-Vásquez R, Buschmann H, Tohme J, Beeching JR (2003) Oxidative stress responses during cassava post-harvest physiological deterioration. Plant Mol Biol 53:669–685

Rentel M, Knight M (2004) Oxidative stress-induced calcium signalling in *Arabidopsis*. Plant Physiol 135:1471–1479

Rickard JE (1981) Biochemical changes involved in the post-harvest deterioration of cassava roots. Trop Sci 23:235–237

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush J (2003) TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34:374–378

Sakai T, Nakagawa Y (1988) Diterpenic stress metabolites from cassava roots. Phytochemistry 27:3769–3779

Sheffield J, Taylor N, Fauquet C, Chen SX (2006) The cassava (*Manihot esculenta* Crantz) root proteome: protein identification and differential expression. Proteomics 6:1588–1598

Showalter A (1993) Structure and function of plant cell wall proteins. Plant Cell 5:9–23

Shyu DJH, Chyan CL, Tzen JTC, Chou WM (2004) Molecular cloning, expression and functional characterization of a cystatin from pineapple stem. Biosci Biotechnol Biochem 68:1681–1689

Solomon M, Belenghi B, Delledonne M, Menachem E, Levine A (1999) The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell 11:431–443

Stec I, Nagl SB, van Ommen GJB, den Dunnen JT (2000) The PWWP domain: a potential protein–protein interaction domain in nuclear proteins influencing differentiation? FEBS Lett 473:1–5

Tanaka Y, Data ES, Hirose S, Taniguchi T, Uritani I (1983) Biochemical changes in secondary metabolites in wounded and deteriorated cassava roots. Agric Biol Chem 47:693–700

Tenhaken R, Thulke O (1996) Cloning of an enzyme that synthesises a key nucleotide sugar precursor of hemicellulose biosyntheis from soybean: UDP-glucose dehydrogenase. Plant Physiol 112:1127–1134

Uritani I, Data ES, Villegas RJ, Flores P, Hirose S (1983) Relationship between secondary metabolism changes in cassava root tissue and physiological deterioration. Agric Biol Chem 47:1591–1598

Wang X, Wang C, Sang Y, Qin C, Welti R (2002) Networking of phospholipases in plant signal transduction. Physiol Plant 115:331–335

Werck-Reichhart D, Bak S, Paquette S (2002) Cytochromes P450. In: Somerville CR, Meyerowitz EM (eds) The *Arabidopsis*

book, American Society of Plant Biologists, Rockville, MD, http://www.aspb.org/publications/arabidopsis/

Wheatley CC, Schwabe WW (1985) Scopoletin involvement in post-harvest physiological deterioration of cassava root (*Manihot esculenta* Crantz). J Exp Bot 36:783–791

Wise M (2003) LEAping to conclusions: a computational reanalysis of late embryogenesis abundant proteins and their possible roles. BMC Bioinformantics 4:52–71

Wong H, Sakamoto T, Kawasaki T, Umemura K, Shimamoto K (2004) Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. Plant Physiol 135:1447–1456

Woo HH, Hawes MC (1997) Cloning of genes whose expression is correlated with mitosis and localized in dividing cells in root caps of *Pisum sativum* L. Plant Mol Biol 35:1045–1051

Zhang P, Bohl-Zenger S, Puonti-Kaerlas J, Potrykus I, Gruissem W (2003) Two cassava promoters related to vascular expression and storage root formation. Planta 218:192–203

Zhang C, Lang P, Dane F, Ebel R, Singh N, Locy R, Dozier W (2005) Cold acclimation induced genes of trifoliate orange (Poncirus trifoliata). Plant Cell Rep 23:764–769