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Judith Owiti, Jonas Grossmann, Peter Gehrig, Christophe Dessimoz, Christophe Laloi, et al.. iTRAQ-based analysis of changes in the cassava root proteome reveals pathways associated with post-harvest physiological deterioration. *The Plant Journal*, 2011, 67 (1), pp.145-156. <10.1111/j.1365-313X.2011.04582.x>. <hal-03034808>

HAL Id: hal-03034808

<https://hal.science/hal-03034808v1>

Submitted on 6 Jan 2023

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iTRAQ-based analysis of changes in the cassava root proteome reveals pathways associated with post-harvest physiological deterioration

Judith Owiti¹, Jonas Grossmann², Peter Gehrig², Christophe Dessimoz³, Christophe Laloi^{1,†}, Maria Benn Hansen¹, Wilhelm Gruissem¹ and Hervé Vanderschuren^{1,*}

¹Department of Biology, Plant Biotechnology, Eidgenössische Technische Hochschule (ETH) Zurich, Universitätstraße 2, 8092 Zurich, Switzerland,

²Functional Genomics Center Zurich, Eidgenössische Technische Hochschule (ETH) Zurich, Winterthurerstraße 190, 8057 Zurich, Switzerland, and

³Department of Computer Science, Eidgenössische Technische Hochschule (ETH) Zurich, Universitätstraße 6, 8092 Zurich, Switzerland

Received 15 November 2010; revised 15 March 2011; accepted 17 March 2011; published online 9 May 2011.

*For correspondence (fax +41 44 632 10 79; e-mail hvanderschuren@ethz.ch).

†Present address: Laboratoire de Génétique et Biophysique des Plantes, iBEB, UMR 6191 CNRS/CEA/Aix-Marseille Université, F-13009 Marseille, France.

SUMMARY

The short storage life of harvested cassava roots is an important constraint that limits the full potential of cassava as a commercial food crop in developing countries. We investigated the molecular changes during physiological deterioration of cassava root after harvesting using isobaric tags for relative and absolute quantification (iTRAQ) of proteins in soluble and non-soluble fractions prepared during a 96 h post-harvest time course. Combining bioinformatic approaches to reduce information redundancy for unsequenced or partially sequenced plant species, we established a comprehensive proteome map of the cassava root and identified quantitatively regulated proteins. Up-regulation of several key proteins confirmed that physiological deterioration of cassava root after harvesting is an active process, with 67 and 170 proteins, respectively, being up-regulated early and later after harvesting. This included regulated proteins that had not previously been associated with physiological deterioration after harvesting, such as linamarase, glutamic acid-rich protein, hydroxycinnamoyl transferase, glycine-rich RNA binding protein, β -1,3-glucanase, pectin methylesterase, maturase K, dehydroascorbate reductase, allene oxide cyclase, and proteins involved in signal pathways. To confirm the regulation of these proteins, activity assays were performed for selected enzymes. Together, our results show that physiological deterioration after harvesting is a highly regulated complex process involving proteins that are potential candidates for biotechnology approaches to reduce such deterioration.

Keywords: post-harvest physiological deterioration, cassava, iTRAQ, crop proteomics, oxidative stress, cell wall remodeling.

INTRODUCTION

Abiotic stress conditions cause extensive losses to agricultural production worldwide (Mittler, 2006). Identification of global gene expression changes in crop genomes upon abiotic stress provides new perspectives to reduce the impact of such stresses on crop production. Cassava (*Manihot esculenta* Crantz) is a tropical plant that is one of the top five staple crops in global production, more than half of it in Africa (<http://faostat.fao.org>). Cassava produces reasonable yields under sub-optimal climatic and agro-ecological conditions, therefore qualifying as a food security crop. One of

the most critical constraints to cassava production, utilization and commercialization is the short shelf-life of its storage roots due to rapid post-harvest physiological deterioration (PPD) (Sanchez *et al.*, 2006). Such deterioration occurs 24–48 h after harvest (Rickard, 1985; Beeching *et al.*, 1998), and starts with vascular streaking, which is a blue/black discoloration of the vascular parenchyma, followed by general discoloration of the storage parenchyma that renders the roots unpalatable. This initial deterioration is purely physiological, and is known as primary PPD. So-called

secondary PPD is attributed to microbial infection, which typically begins within 5–7 days after harvesting (O'Brien *et al.*, 1995). Consequently, the roots must be consumed immediately after harvesting, which limits marketing options for cassava to locations close to production sites.

Molecular investigations of PPD have been limited to a few molecular pathways as well as gene expression profiling studies. These studies have shown that PPD in cassava root is initiated by an oxidative burst (Reilly *et al.*, 2004), and is followed by accumulation of secondary metabolites (Buschmann *et al.*, 2000; Bayoumi *et al.*, 2008a,b). The initial oxidative burst that occurs within 15 min of the root being injured is characterized by early up-regulation of genes involved in turnover of reactive oxygen species (ROS) (Reilly *et al.*, 2001, 2004). Programmed cell death (PCD) activation has also been hypothesized on the basis of two independent transcript analysis studies based on up-regulation of protease, chitinase and PCD inhibitor genes (Huang *et al.*, 2001; Reilly *et al.*, 2007). Cycloheximide inhibition of protein synthesis indicated that PPD is an active process involving changes in gene expression as well as protein synthesis (Beeching *et al.*, 1998). Although these studies have increased our understanding of PPD, the approaches were restricted to elements of the process that are regulated at the transcript level. Thus, equally important information on regulation at the protein level is currently missing. Because transcript accumulation does not always correlate with protein accumulation (Resch *et al.*, 2006; Gallardo *et al.*, 2007; Baerenfaller *et al.*, 2008; Van Der Kelen *et al.*, 2009), proteomics studies are necessary to understand PPD at the protein level.

Previously reported cassava proteomics studies were limited to 2D gel electrophoresis analysis of roots and somatic embryos (Sheffield *et al.*, 2006; Baba *et al.*, 2008). However, the 2D gel technique has several limitations, which limits its application for comprehensive analysis of proteome changes (Wienkoop and Weckwerth, 2006; Schuize and Usadel, 2010). Although most proteomics studies in crop plants have used 2D gel approaches (Salekdeh and Komatsu, 2007), alternative methods are now available. In-solution peptide labeling combined with LC/MS has emerged as a robust approach for protein profiling studies (Zieske, 2006; Gstaiger and Aebersold, 2009). In this study, we successfully used isobaric tags for relative and absolute quantification (iTRAQ) (Ross *et al.*, 2004) to assess proteome changes in cassava root during PPD. We took advantage of the multiplex protein profiling capability of the iTRAQ technology to perform a time-course characterization of cassava root proteome during onset of PPD. Because of the extended protein coverage and improved differential extraction procedures, our work integrates comprehensive information on the cassava root proteome with identification of differentially expressed proteins involved in PPD modulation.

Although cassava cDNA microarray technology was developed using EST sequence information available in databases (Lopez *et al.*, 2005; Reilly *et al.*, 2007), proteomics studies of organisms with unsequenced or partially sequenced genomes rely on protein databases comprising single protein sequences from many plant species. Such databases often limit protein identifications (Grossmann *et al.*, 2007). As knowledge of the cassava genome sequence and annotation was limited at the time of this study, we developed a bioinformatics approach to specifically process cassava proteomics data. The proposed workflow is also suitable for proteome analysis of other plants with unsequenced genomes, because identified proteins are converted into a format that allows the use of tools developed for the model plant *Arabidopsis thaliana*. Our approach significantly improved the analysis and understanding of the pathways involved in PPD. The results revealed proteins in cassava roots that are significantly modulated during onset of PPD, allowing us to extend the current PPD model. The regulation of protein levels of selected candidate enzymes identified in the iTRAQ study was confirmed by enzymatic assays.

RESULTS AND DISCUSSION

Protein identification from cassava root

To combine our large-scale cassava root proteome analysis with quantitative information on proteins modulated during PPD, we used isobaric tags for relative and absolute quantification (iTRAQ) (Ross *et al.*, 2004; Zieske, 2006). The expression profiles of soluble and non-soluble cassava root proteins at early PPD (6, 12 and 24 h) and late PPD (48, 72 and 96 h) were analyzed in two independent biological experiments (Figure 1). We used the *Viridiplantae* protein database to identify cassava root proteins because the cassava draft genome sequence was not publicly available at the time of the study. After merging data from the two experiments, 8960 proteins were identified with a confidence cutoff of $\geq 90\%$. A total of 8264 proteins were identified with a

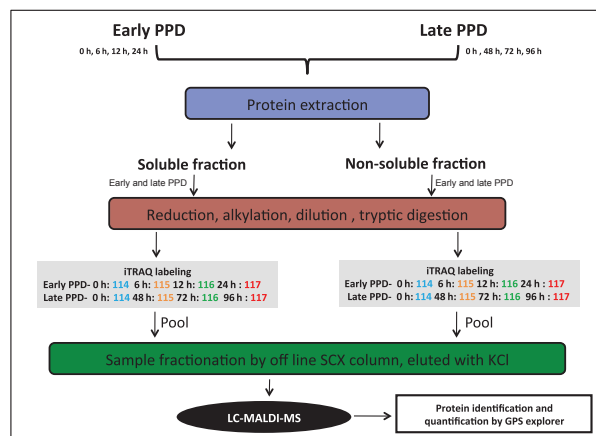


Figure 1. General workflow of the cassava iTRAQ experiment.

confidence cut-off of $\geq 95\%$ in the two biological replicates: 5257 proteins from experiment 1 and 3007 proteins from experiment 2 (Table S1). Using representative datasets from experiments 1 and 2, searches were also performed against a decoy (reversed) database, leading to estimation of false discovery rates of 3.22% and 2.57%, respectively (Table S2) (Käll *et al.*, 2008). Detected peptides are usually searched against a protein reference database or gene sequence information. Redundancy and homology between proteins listed in databases often make it difficult to assign peptides to specific unique proteins (Podwojski *et al.*, 2010). Because of the presence of homologous proteins, alternative splicing forms or redundant protein entries in the database, an identified peptide may correspond to multiple entries in the protein database (Lu *et al.*, 2009), which often leads to over-estimation of the number of identified proteins. To address the issue of redundancy, the identified proteins were clustered into groups of homologs by aligning all proteins and recursively grouping proteins with highly significant alignment scores (E -value $< 1e-20$) (Karlin and Altschul, 1990). Using this approach with our raw dataset, we generated 1387 non-redundant protein groups, of which approximately 40% (555) were identified in both biological replicates (Figure S1 and Table S3). Six hundred and ten protein groups were unique to experiment 1, and 222 protein groups were unique to experiment 2 (Figure S1). Shotgun proteome identification has been shown to affect the reproducibility of protein identification in replicate iTRAQ experiments (Chen *et al.*, 2007; Gan *et al.*, 2007). The overlap between the two independent biological replicates in our study was comparable to reported studies from organisms with sequenced genomes (Berg *et al.*, 2006; Dunkley *et al.*, 2006; Chen *et al.*, 2007; Datta *et al.*, 2010). Extraction of non-soluble proteins from the sample pellet increased the number of detected proteins (Figure S1). A total of 498 protein groups were unique to the soluble protein fraction, while 387 protein groups were found only in the membrane fraction. There was an overlap of 502 protein groups (36.2%) between the soluble and membrane fractions.

Converting the identified cassava proteins to Arabidopsis orthologs allowed us to use Arabidopsis functional genomics tools for cassava proteome analysis. *Arabidopsis thaliana* orthologs of the identified proteins were determined by aligning each protein sequence against all Arabidopsis amino acid sequences available. Of the 8960 proteins identified in this study, 1110 proteins could be mapped onto *Arabidopsis thaliana* orthologs corresponding to unique proteins (Table S3). There were 711 proteins for which no Arabidopsis orthologs could be identified (Table S3). These proteins did not exhibit a significant match with any Arabidopsis protein, implying that they either do not exist in Arabidopsis or that their amino acid sequences are poorly conserved within the plant kingdom. Conversion of the identified cassava proteins to Arabidopsis orthologs allowed

us to address the protein redundancy encountered when using databases that include protein data from several species. While iTRAQ detects a large number of proteins, it is also more sensitive to redundancy, because peptides originating from a single protein can generate distinct identifications in the database. Further analysis to identify and quantify proteins that are regulated during cassava PPD was therefore performed using the Arabidopsis orthologs list. This approach allowed us to approach true estimation by removing the redundancy inherent in peptide-based identification in unsequenced organisms.

PPD generates important changes in expression of the cassava proteome

Although several published reports of regulated proteins are based on a 1.2–1.5-fold change threshold (Zhu *et al.*, 2009), we used a twofold cut-off to assess changes in abundance as significant for regulated proteins because this cut-off exceeded the maximum false-positive abundance changes in preliminary iTRAQ experiments. Because our dataset included redundant proteins, we used a mean \log_2 ratio for significantly regulated proteins that mapped onto the same *Arabidopsis thaliana* orthologs. Analysis of the cassava proteome dataset revealed significant regulation of 208 of the 1110 proteins at one or more of the early PPD time points. Of these, 67 proteins were up-regulated and 141 proteins were down-regulated. The data set from late PPD samples revealed 349 regulated proteins, of which 179 were up-regulated and 170 were down-regulated. Interestingly, 48 proteins were down-regulated and 20 proteins were up-regulated during both early and late PPD. Tables 1 and 2 give partial lists of the proteins that are significantly regulated during early and late PPD, and the full list of proteins is provided in Table S4.

To examine the overall dynamics of PPD-induced proteome changes in cassava root, we used a newly incorporated feature in MAPMAN (Usadel *et al.*, 2009) that allows organization of proteins into clusters based on their expression patterns. Using the Arabidopsis ortholog list, six expression patterns were selected as most illustrative and informative for the overall PPD-induced proteome changes (Figure S2 and Table S5). It is interesting to note that, at the proteome level, the number of down-regulated proteins is twice as high as the number of up-regulated proteins. This trend was in contrast to transcriptome changes analyzed during early PPD (12 and 24 h), during which more up-regulated than down-regulated transcripts were identified (Huang *et al.*, 2001; Reilly *et al.*, 2007). This difference may reflect increased protein degradation or inhibition of translation during stress (reviewed by Yamasaki and Anderson, 2008).

Extension of the PPD model revealed by iTRAQ analysis and identification of pathways related to PPD

To obtain an overview of the regulated proteins, we generated a master table (Table S6) that summarizes the

Table 1 A selection of proteins found to be regulated during early PPD

Group ID	Protein name and species	Accession number	Peptide count	Best ion score CI (%)	iTRAQ ratio (115/114)	iTRAQ ratio (116/114)	iTRAQ ratio (117/114)	Arabidopsis ortholog
Down-regulated proteins								
782	Histone H2B.11, <i>Arabidopsis thaliana</i>	P40283IH2B11_ARATH	3	100	0.0314	0.0255	0.031	AT5G59910
460	Isocitrate lyase, <i>Ostreococcus tauri</i>	Q01FS8IQ01FS8_OSTTA	1	96.4	0.1621	0.2218	0.2051	AT2G43180
247	Glutathione peroxidase (fragment), <i>Betula verrucosa</i>	Q9M3T7IQ9M3T7_BETVE	1	94.42	0.2144	0.19	0.2173	AT4G31870
141	Glycine-rich RNA-binding protein, <i>Euphorbia esula</i>	O48567IO48567_EUPES	2	100	0.7363	0.8525	0.2092	AT5G61030
188	Dehydrin, <i>Populus nigra</i>	A7L2U4IA7L2U4_POPNI	2	99.98	0.6118	0.4997	0.1138	AT1G76180
38	Ascorbate peroxidase APX3, <i>Manihot esculenta</i>	Q52QX1IQ52QX1_MANES	9	100	1.3528	0.9674	0.2678	AT4G35970
74	Peroxidase, <i>Linum usitatissimum</i>	Q43782IQ43782_LINUS	1	99.97	0.2147	0.338	0.2401	AT5G67400
293	Aquaporin, <i>Prunus persica</i>	A5A8K8IA5A8K8_PRUPE	1	98.11	0.2565	0.3898	0.3584	AT5G60660
107	Phospholipase D, <i>Solanum lycopersicum</i>	Q9FR61IQ9FR61_SOLLC	3	99.98	0.7973	0.5708	0.3754	AT5G25370
1354	Metallothionein 2, <i>Plantago major</i>	Q5ZF75IQ5ZF75_PLAMJ	1	99.31	0.7302	1.1456	0.4308	AT5G02380
44	Serine hydroxymethyltransferase 1, <i>Flaveria pringlei</i>	P49357IGLYM_FLAPR	1	99.38	1.1602	0.7407	0.2254	AT5G26780
139	18.5 kDa class I heat shock protein, <i>Glycine max</i>	P05478IHSP16_SOYBN	5	99.93	0.6444	0.5986	0.249	AT5G59720
384	S-adenosyl-L-methionine synthetase (fragment), <i>Chlamydomonas incerta</i>	Q1WLU2IQ1WLU2_CHLIN	3	100	0.3126	0.8952	0.4923	AT4G01850
Up-regulated proteins								
245	Calmodulin-1/11/16, <i>Daucus carota</i>	P62200ICALM1_DAUCA	5	100	2.211	2.0394	2.2607	AT5G42380
16	12-oxophytodienoate reductase, <i>Hevea brasiliensis</i>	Q4ZJ73IQ4ZJ73_HEVBR	2	100	1.0051	1.4917	2.2321	AT2G06050
286	β -1,3-glucanase (fragment), <i>Manihot esculenta</i>	A0SVL9IA0SVL9_MANES	2	100	1.2818	2.2491	1.6846	AT5G64790
640	Quinone oxidoreductase, <i>Arabidopsis thaliana</i>	Q9FKG8IQ9FKG8_ARATH	1	100	1.8679	2.7333	2.2381	AT5G61510
266	Glutamic acid-rich protein, <i>Manihot esculenta</i>	Q6XQ13IQ6XQ13_MANES	5	100	3.0873	2.2243	1.746	n/a
237	14-3-3-like protein, <i>Gossypium hirsutum</i>	A1XJ43IA1XJ43_GOSHI	4	100	2.3775	2.9032	2.7335	AT5G65430
266	Allergenic-related protein Pt2L4, <i>Manihot esculenta</i>	Q8L5H5IQ8L5H5_MANES	5	100	3.1008	3.014	2.0111	n/a
224	Linamarase, <i>Manihot esculenta</i>	Q41172IQ41172_MANES	1	99.99	3.2138	2.4315	1.1453	AT5G54570
163	Phenylalanine ammonia lyase 2, <i>Manihot esculenta</i>	Q94F89IQ94F89_MANES	3	100	1.1708	3.6907	3.7366	AT5G04230
251	Superoxide dismutase [Cu-Zn], <i>Brassica juncea</i>	Q8H6F2IQ8H6F2_BRAJU	1	100	1.2132	3.9559	1.9116	AT5G18100
163	Phenylalanine ammonia lyase 1 (fragment), <i>Manihot esculenta</i>	Q94F90IQ94F90_MANES	4	100	1.2655	3.5264	4.8407	AT5G04230
348	Hydroquinone glucosyltransferase, <i>Rauvolfia serpentina</i>	Q9AR73IHQGT_RAUSE	1	99.03	1.8187	4.4863	4.2037	AT5G66690
65	Putative pectin methylesterase, <i>Populus tremula</i> \times <i>Populus tremuloides</i>	Q9FY03IQ9FY03_9ROSI	2	100	1.5603	1.9427	2.0436	AT5G64640
92	Maturase K, <i>Castanopsis inermis</i>	Q75VC1IQ75VC1_9ROSI	1	98.34	1.5342	1.8477	2.0343	n/a

n/a, not available.

change ratios of the proteins in the previously identified protein groups. The master table was useful in relating proteins to biochemical and physiological functions that

are modulated during the onset of PPD. Some of the regulated proteins in the most important pathways are shown in Figure 2.

Table 2 A selection of proteins found to be regulated during late PPD

Group ID	Protein and species	Accession number	Peptide count	Best ion score CI %	iTRAQ ratio (115/114)	iTRAQ ratio (116/114)	iTRAQ ratio (117/114)	Arabidopsis ortholog
Down regulated proteins								
795	Histone H3, <i>Arabidopsis thaliana</i>	A6QRD3IA6QRD3_ARATH	3	99.6	0.0463	0.0296	0.0392	AT5G65360
494	Sulfite reductase (fragment), <i>Allium cepa</i>	Q5IEZ9IQ5IEZ9_ALLCE	1	100	0.2182	0.0445	0.0708	AT5G04590
188	Dehydrin, <i>Populus nigra</i>	A7L2U4IA7L2U4_POPNI	1	99.994	0.0882	0.0934	0.1105	AT1G76180
29	Glutathione transferase (fragment), <i>Brassica juncea</i>	A1Z283IA1Z283_BRAJU	1	99.948	0.0923	0.1064	0.9172	AT5G17220
107	Phospholipase D α , <i>Fragaria ananassa</i>	Q533V0IQ533V0_FRAAN	3	100	0.4039	0.1326	0.1262	AT5G25370
293	Aquaporin, <i>Prunus persica</i>	A5A8K8IA5A8K8_PRUPE	1	99.764	0.1735	0.1939	0.2243	AT5G60660
232	Dehydroascorbate reductase (fragment), <i>Solanum tuberosum</i>	Q1G149IQ1G149_SOLTU	1	100	0.148	0.2194	0.0975	AT5G36270
141	Glycine-rich RNA-binding protein, <i>Euphorbia esula</i>	O48567IO48567_EUPES	2	100	1.0951	0.52	0.2721	AT5G61030
544	ACC oxidase ACCO2, <i>Manihot esculenta</i>	Q52QX6IQ52QX6_MANES	2	100	0.4842	0.2468	0.1901	AT5G63600
206	Allene oxide cyclase C1, <i>Humulus lupulus</i>	Q68IP7IQ68IP7_HUMLU	2	100	0.3812	0.2632	0.2265	AT3G25780
169	Thioredoxin-dependent peroxidase, <i>Nelumbo nucifera</i>	A3FPF4IA3FPF4_NELNU	3	99.998	0.3984	0.3981	0.1719	AT3G52960
38	Ascorbate peroxidase APX3, <i>Manihot esculenta</i>	Q52QX1IQ52QX1_MANES	2	100	0.3246	0.2634	0.4335	AT4G35970
282	Polygalacturonase-inhibiting protein, <i>Cucumis melo</i>	Q7X9Q7IQ7X9Q7_CUCME	1	100	0.4743	0.3644	0.3008	AT5G67280
164	Late embryogenesis-like protein (fragment), <i>Prunus armeniaca</i>	O81366IO81366_PRUAR	1	98.873	0.5482	0.44	0.6073	AT2G44060
415	Δ^1 -pyrroline-5-carboxylate dehydrogenase, <i>Arabidopsis thaliana</i>	Q8VZC3IAL121_ARATH	8	99.999	1.0169	0.4635	0.5652	AT5G62530
Up-regulated proteins								
285	Catalase isozyme (fragment), <i>Triticum aestivum</i>	Q7XYE2IQ7XYE2_WHEAT	2	99.99	1.3655	2.0227	2.0033	AT4G35090
266	Allergenic-related protein Pt2L4, <i>Manihot esculenta</i>	Q8L5H5IQ8L5H5_MANES	4	100	2.0149	1.4329	0.9304	n/a
92	Maturase K (fragment), <i>Rhodosciadium nudicaule</i>	O20081IO20081_9APIA	1	99.95	1.87	2.1815	2.0346	n/a
16	12-oxophytodienoate reductase, <i>Hevea brasiliensis</i>	Q4ZJ73IQ4ZJ73_HEVBR	2	99.93	1.9806	2.2739	1.8475	AT2G06050
245	Calmodulin (fragment), <i>Solanum lycopersicum</i>	O24033IO24033_SOLLC	4	100	0.7736	2.3169	1.227	AT5G37780
76	Flavonoid 3'-hydroxylase, <i>Lobelia erinus</i>	A4F1U3IA4F1U3_LOBER	1	96.87	3.0797	2.4247	2.1703	AT5G67310
65	Putative pectin methylesterase, <i>Populus tremula</i> \times <i>Populus tremuloides</i>	Q9FY03IQ9FY03_9ROSI	2	100	2.233	2.5094	2.9649	AT5G64640
353	Hydroxycinnamoyl transferase, <i>Nicotiana tabacum</i>	Q8GSM7IQ8GSM7_TOBAC	1	99.82	1.0618	2.2383	3.0617	AT5G67160
288	Cysteine proteinase inhibitor, <i>Ricinus communis</i>	Q43635IQ43635_RICCO	2	99.98	1.4793	2.5827	2.9803	AT5G12140
142	Xyloglucan endotransglucosylase XET2, <i>Capsicum annuum</i>	Q1W4A2IQ1W4A2_CAPAN	1	99.31	3.5005	2.9522	1.9848	AT5G65730
163	Phenylalanine ammonia lyase 1, <i>Glycine max</i>	P27991IPAL1_SOYBN	5	99.89	3.5988	2.6315	3.6875	AT5G04230

Table 2 (Continued)

Group ID	Protein and species	Accession number	Peptide count	Best ion score CI %	iTRAQ ratio (115/114)	iTRAQ ratio (116/114)	iTRAQ ratio (117/114)	Arabidopsis ortholog
358	Cellulose synthase, <i>Populus tremula</i> × <i>Populus tremuloides</i>	Q6J8X1 Q6J8X1_9ROSI	1	95.86	5.0944	3.5762	3.0356	AT5G64740
286	β-1,3-glucanase (fragment), <i>Manihot esculenta</i>	A0SVL9 A0SVL9_MANES	3	100	1.7863	3.3286	6.1767	AT5G64790
224	Linamarase, <i>Manihot esculenta</i>	Q41172 Q41172_MANES	1	100	6.6003	3.8079	4.9489	AT5G54570

n/a, not available.

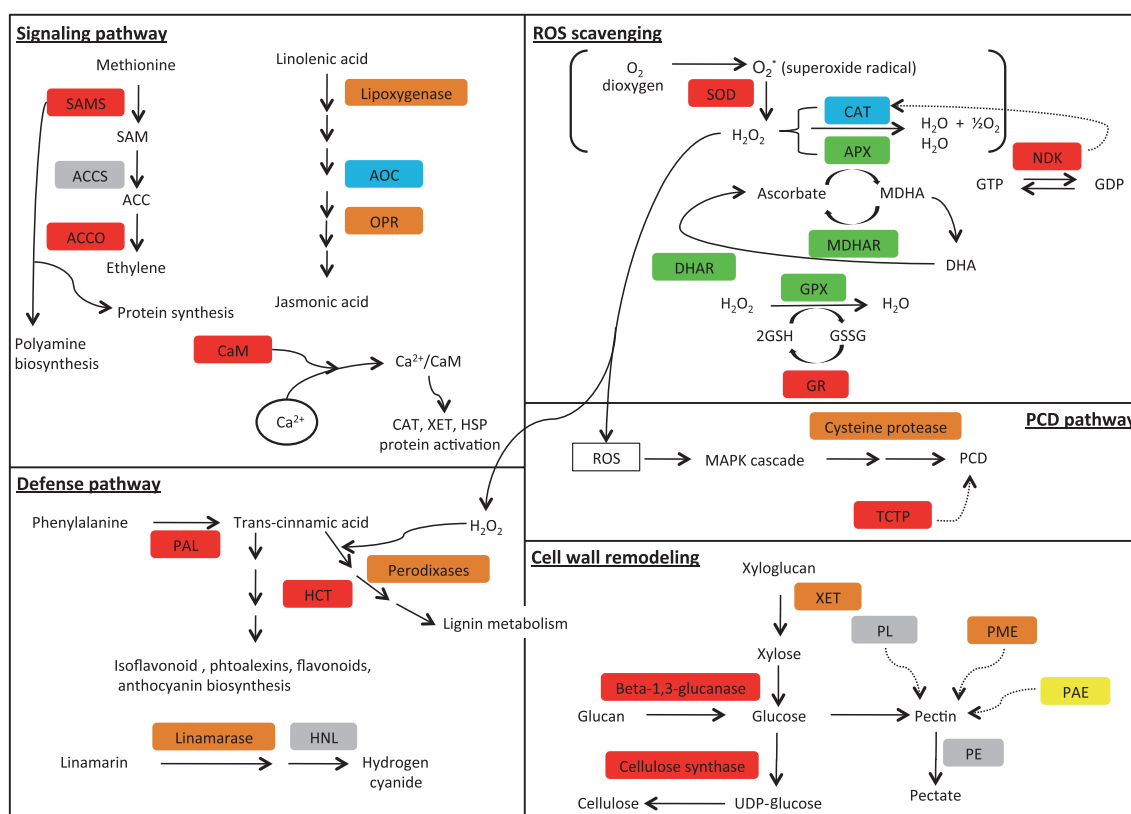


Figure 2. Summary of some of the biological pathways affected during PPD in cassava.

Red boxes indicate proteins up-regulated during early PPD, brown boxes indicate proteins up-regulated during late PPD, yellow boxes indicate proteins down-regulated during early PPD, and green boxes represent proteins down-regulated during late PPD. The blue boxes represent proteins that were shown to have mixed expression patterns (both up- and down-regulation). SAMS, S-adenosyl methionine synthase; ACCS, 1-aminocyclopropane-1-carboxylate synthase; ACCO, 1-aminocyclopropane-1-carboxylate oxidase; AOC, allene oxide synthase; AOC, allene oxide cyclase; OPR, oxophytodienoate reductase; CaM, calmodulin; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; NDK, nucleoside diphosphate kinase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GPX, glutathione peroxidase; GR, glutathione reductase; TCTP, translationally controlled tumor protein; PAL, phenylalanine ammonia lyase; HCT, hydroxycinnamoyl transferase; HNL, hydroxynitrile lyase; XET, xyloglucan endotransglycosylase; PL, pectate lyase; PME, pectin methyl esterase; PAE, pectin acetyltransferase; PE, pectin esterase.

ROS scavenging pathways. Our cassava root proteome analysis confirms the PPD-associated increase in gene expression for enzymes involved in ROS turnover (i.e.

catalases [CAT] and glutathione-S-transferase [GST]), as well as programmed cell death (PCD). Other key enzymes, such as nucleoside diphosphate kinase (NDK) and super-

oxide dismutase (SOD), were also up-regulated at the protein level, although early studies based on microarray results concluded that SOD was not activated during PPD (Reilly *et al.*, 2007). However, our analysis revealed that both Mn SOD and Cu/Zn SOD enzyme levels were increased in the soluble protein fractions. Ascorbate peroxidase 3 (APX3) showed consistent down-regulation but was not previously detected in the microarray analysis. Our bioinformatic analysis (data not shown) later confirmed that the APX3 accession corresponds to the *Manihot esculenta* APX1 protein. NDK was recently reported to be involved in ROS resistance and to interact with CAT1 (Haque *et al.*, 2010).

PCD pathway. The PCD pathway appeared to be activated, with a consistent increase of cysteine proteases during late PPD. Interestingly, many peptides of the phospholipase D α -group of enzymes were down-regulated during early and late PPD time points. The plant phospholipase D family is relatively large and heterogeneous, and various phospholipase D isoforms are involved in separate stress responses by activating signal transduction cascades and modifying biophysical properties of lipid membranes (Bargmann and Munnik, 2006).

Defense pathways. Linamarase, a β -glucosidase, has been particularly studied in cassava because of its function in cyanogenesis. The enzyme is primarily located in the cell wall and in vesicle-like structures in latex (Pancoro and Hughes, 1992; Santana *et al.*, 2002). We detected linamarase in the non-soluble protein fraction, and found that it was consistently up-regulated during late PPD time points (group 224, Figure 2 and Table S6). Our results show that linamarase can be synthesized *de novo* in cassava roots, revising earlier models indicating that linamarase is synthesized in cassava leaves and transported to the roots through the branched laticifer network (Pancoro and Hughes, 1992; Santana *et al.*, 2002). The function of β -glucosidases is well understood in defense responses against pathogens (Buschmann *et al.*, 2000), and their up-regulation can be triggered by hormones such as methyl jasmonate (Stenzel *et al.*, 2003a,b). The up-regulation of linamarase during late PPD may therefore be a response to the microbial colonization that usually occurs after the onset of PPD (Stotz *et al.*, 2000).

Signaling pathways. Strikingly, proteins involved in jasmonic acid (JA) biosynthesis were also modulated during PPD, including lipoxygenase, allene oxide cyclase (AOC) and 12-oxophytodienoate reductase (OPR). While lipoxygenase and OPR were up-regulated during late PPD, detection and quantification of AOC-derived peptides revealed a mixed pattern, suggesting that members of the AOC gene family are regulated differently during PPD. Arabidopsis has four AOC genes (Stenzel *et al.*, 2003b; Glauser *et al.*, 2008), and

analysis of the genome sequence reveals that cassava has at least three genes encoding AOC. The JA pathway is typically activated shortly after wounding (Ravanel *et al.*, 1998), and its activation during PPD reveals similarities to wound response.

A methionine synthase was consistently up-regulated starting 12 h after the onset of PPD. Methionine has a central function in cellular metabolism, and is required for protein synthesis and methyl group transfer through S-adenosyl-methionine (AdoMet), as well as polyamine and ethylene synthesis. Activation of the methionine pathway is consistent with up-regulation of ethylene biosynthesis enzymes during PPD.

Gene expression of Ca^{2+} sensors such as calmodulin (CaM) is often induced by various abiotic stresses (Snedden and Fromm, 2001). We found a significant up-regulation of calmodulin proteins during early PPD (group 245, Table S6). The initial up-regulation of CaM may be caused by a rapid increase in Ca^{2+} (Kaplan *et al.*, 2006) resulting from an oxidative burst early in PPD (Grant *et al.*, 2000; Rentel and Knight, 2004). Kaplan *et al.* (2006) identified XET as a Ca^{2+} -responsive up-regulated gene. The Ca^{2+} -CaM complex binds and activates a collection of target proteins leading to a physiological response (Zielinski, 1998; Bouche *et al.*, 2005). Expression of CaM plays an important signal transduction role during heat stress, and expression of several heat shock proteins (HSPs) has been correlated with accumulation of CaM transcripts and proteins in plants (Liu *et al.*, 2003; Zhang *et al.*, 2009). However, we found that the levels of small heat shock proteins (sHSPs) tended to decline during PPD (group 260, Table S6). The iTRAQ measurements also revealed a mixed pattern for accumulation of HSP70 proteins, but, because of the strong conservation of HSP70 superfamily member sequences, it was difficult to discriminate the regulation of specific family members.

Cell-wall metabolism. A subset of cell wall-modifying proteins was found to be up-regulated during PPD, including xyloglucan endotransglycosylase (XET), pectin methyl esterase, hydroxycinnamoyl transferase and cellulose synthase. The up-regulation of cellulose synthase in early and late PPD, combined with XET up-regulation in late PPD, suggests that the cell wall undergoes significant remodeling during PPD. XET activities, the cellulose/xyloglucan ratio and cellulose-xyloglucan interactions are important parameters that determine the mechanical and growth properties of the cell wall (Chanliaud *et al.*, 2004; Cosgrove, 2005).

We also found that the protein levels of hydroxycinnamoyl transferase (HCT) and phenylalanine ammonia lyase (PAL) were up-regulated. These enzymes function in the phenylpropanoid pathway (Hoffmann *et al.*, 2004), suggesting that their up-regulation may affect lignin content during early and late PPD. Lignin-forming peroxidases were also

strongly up-regulated during late PPD. Both biotic and abiotic stresses have been reported to affect lignin content in plants (Moura *et al.*, 2010). Pectin methyl esterase (PME) was also up-regulated during late PPD. PME peptides were mostly detected in the non-soluble fraction (Table S6). Down-regulation of pectin acetyltransferase (PAE) during early PPD and up-regulation during late PPD (group 173, Table S6) reinforces the view that the cassava root cell wall undergoes remodeling through pectin modifications during PPD.

Other pathways. Expression of maturase K has so far been reported only in green plant tissues. Maturase K is involved in plant development and photosynthesis (Barthet and Hilu, 2007) as well as intron splicing (Zoschke *et al.*, 2010). We detected the protein in cassava roots (group 92, Table S6), in which it is strongly up-regulated during PPD, consistent with PPD-associated gene expression changes. We also detected modulation of several other proteins whose role in PPD remains unclear. For example, the glutamic acid-rich protein Pt2L4 (group 266, Table S6) was up-regulated during early PPD and then down-regulated during late PPD. The Pt2L4 transcript was previously reported to accumulate in roots (Zhang *et al.*, 2003) and is differentially expressed during root development (de Souza *et al.*, 2009).

The activity of identified enzymes is regulated during PPD

To confirm the role of identified cassava proteins whose accumulation was modulated during PPD, we selected enzymes whose activity could be measured using established assays. We used both soluble and non-soluble protein fractions in which enzyme levels were modulated according to the iTRAQ results. APX, SOD and β -1,3-glucanase activities were measured using soluble protein fractions, while PME activity was measured in non-soluble protein fractions.

APX activity gradually increased between 12 and 96 h (Figure 3a), with significant activation during late PPD. This activation does not follow the reported transcript accumulation (Reilly *et al.*, 2004, 2007) or our own RNA measurements (data not shown), which show that APX mRNA levels increase only during early PPD. It is also not consistent with the protein abundance based on APX peptide quantification, which declines later during PPD (see Table S6). However, the transcript and proteomics data are based on the detection of a restricted number of APX isoforms, and therefore we cannot exclude the possibility that other undetected APX isoforms contribute to the enzyme activity. The soluble protein extracts that we used in the activity assay most likely contained cytosolic, peroxisomal, mitochondrial and plastidial APX isoforms, which may have different activities compared to the cytosolic isoforms detected in the iTRAQ analysis. APX activity generally increases together with the activities of other antioxidant enzymes, such as catalase, SOD and glutathione reductase, in response to various stresses, suggesting that enzymes involved in ROS scav-

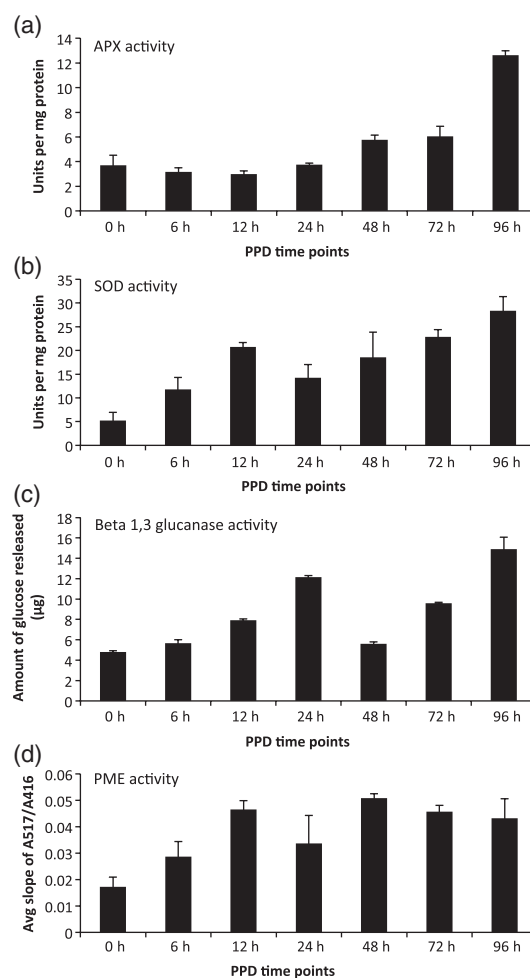


Figure 3. Enzymatic assays. (a) Ascorbate peroxidase enzyme activity. (b) Superoxide dismutase activity. (c) β -1,3-glucanase activity. (d) Pectin methyl esterase activity.

enging are co-regulated (Shigeoka *et al.*, 2002). We found a general increase of SOD protein levels in the soluble fraction, with peaks at 12, 72 and 96 h. SOD activity increased from 0 to 12 h, and then further between 24 and 96 h (Figure 3b). Transcript (data not shown) and protein data (Table S6) showed an increase at 12 h followed by a decrease at 24 h. In a previous study on oxidative stress, Reilly *et al.* (2004) did not detect changes in SOD protein levels during onset of PPD. This may be due to their use of gel-based and visual methods to evaluate SOD activities. The modulation of SOD-derived peptides in our iTRAQ study reflected changes of SOD activity upon PPD.

As discussed above, our iTRAQ analysis also showed that accumulation of cell wall-modifying enzymes was modulated during PPD. To confirm these results, we measured the activities of β -1,3-glucanase and pectin methylesterase. β -1,3-glucanase activity steadily increased in the soluble

fraction of the protein extracts prepared between 0 and 24 h, followed by a sharp decline in activity between 24 and 48 h (Figure 3c). β -1,3-glucanase activity increased again between 48 and 96 h, with the activity peaking at 96 h. The enzyme activity pattern of β -1,3-glucanase and SOD was similar to the co-regulation of SOD and glucanase at the transcript level during chilling injury of grapefruit (*Citrus paradisi*) (Sapitnitskaya *et al.*, 2006). The two-peak pattern of β -1,3-glucanase and SOD activities was consistent with their protein modulation pattern (Table S6).

Pectin methylesterase contributes to cell-wall loosening and degradation by promoting the activity of cell-wall hydrolases such as endopolygalacturonases (Micheli, 2001; Willats *et al.*, 2001). PME protein abundance increased gradually from early to late PPD (Table S6), although the increase was significant only between 24 and 96 h. The enzymatic assay revealed a similar pattern, with a sustained high PME activity level between 12 and 96 h (Figure 3d). PMEs belong to a large multi-gene family, and differential transcript regulation has been reported within the family when plants are subjected to biotic and abiotic stresses (Pelloux *et al.*, 2007). While it is difficult to discriminate the modulation of each PME by the proteomics approach, global PME up-regulation could be detected in late PPD by iTRAQ measurements. The enzymatic assays provided additional evidence that regulation and activity of PMEs are linked to the onset of PPD.

Together, the enzymatic assays confirmed that the iTRAQ analysis is a suitable proxy for enzymes that are differentially regulated during onset of PPD. Our results also show that enzyme activity assays should be combined with quantitative proteome analysis to determine the regulation of enzyme activities at translational or post-translational levels.

CONCLUSION

Our results show that iTRAQ is a robust method to detect and quantify a large number of cassava proteins, including some proteins from the membrane fraction. Furthermore, we identified a large number of differentially expressed proteins involved in both the onset and late stages of PPD in cassava roots. Our quantitative proteomics approach revealed modulation of signaling pathways (i.e. calmodulin and jasmonic acid) and regulation of important enzymes such as linamarase and PME that were not previously known to be associated with PPD. Strong correlations between enzymatic activities and protein levels for some of the modulated proteins (e.g. SOD, β -1,3-glucanase and PME) established the basis for their regulation. Collectively, our results represent the most comprehensive proteome coverage of the cassava root in response to PPD. These results significantly increase our understanding of molecular processes involved in cassava PPD, and have identified additional target proteins that are candidates for biotechnology approaches to delay or reduce PPD.

EXPERIMENTAL PROCEDURES

Plant material

Commercial waxed roots of medium size, 16–18 cm long, were selected for the study. The storage roots were sliced and layered on Petri dishes containing filter papers pre-soaked with 2 ml of distilled water to prevent drying. The sliced roots were then randomized and stored at a constant temperature of 27°C in the dark. Slices were taken and frozen in liquid nitrogen after incubation for 0, 6, 12, 24, 48, 72 or 96 h.

Protein extractions

Prior to blending, 20 ml of protein extraction buffer (20 mM HEPES buffer, pH 8, 5 mM $MgCl_2$, one tablet of EDTA-free protease inhibitor per 50 ml buffer) was added to 100 g of cassava peeled cassava root slices. Following centrifugation (4000 g, 4°C, 20 min), the supernatant was used for soluble protein extraction. The non-soluble protein fraction was obtained from the pellet derived from the initial extraction using the same extraction buffer with 3% CHAPS.

Reduction, denaturation and blocking of cysteine residues

Proteins were dissolved in 100 mM triethylammonium bicarbonate buffer at pH 8.5. Samples were normalized based on protein concentration. Protein reduction, denaturation and blocking of cysteine residues were performed according to the manufacturer's protocol using the reagents provided (Applied Biosystems, <http://www.appliedbiosystems.com/>). Samples weighing 100 μ g were treated with 2 μ l of the reducing agent Tris(2-carboxyethyl) phosphine (TCEP) at 60°C for 1 h, and alkylated using 1 μ l of the cysteine blocking reagent methyl methanethiosulfonate for 10 min at room temperature.

Protein digestion

Protein samples were digested using sequencing grade trypsin (Promega, <http://www.promega.com/>) at a ratio of 1:10 (w:w) for 16 h at 37°C. Peptides from each sample in a final volume of 50 μ l were labeled with one of the four iTRAQ reagents at room temperature.

Labeling the protein digests with the iTRAQ reagents

Labeling of the samples (100 μ g) with the 4-plex iTRAQ reagents (Applied Biosystems) was performed according to the manufacturer's recommendations. Samples taken at 0, 6, 12 and 24 h (early PPD) and 48, 72 and 96 h (late PPD) were each labeled with iTRAQ reagents with molecular masses of 114, 115, 116, and 117 Da. After labeling, all four samples were combined, and acidified to pH 3 using phosphoric acid.

Strong cation exchange separation of peptides (HPLC)

After labeling tryptic digests from the various samples, the peptides were diluted with buffer A (10 mM potassium phosphate buffer, pH 2.85, 25% acetonitrile) at a 1:3 ratio (v:v), and fractionated by strong cation exchange (SCX) chromatography on a poly-sulfoethyl A column (PolyLC, <http://www.polylc.com>) (100 \times 2.1 mm, 5 μ m particles with 300 Å pores) using an Agilent 1100 HPLC system (<http://www.agilent.com>) connected to a fraction collector. Twenty-four SCX fractions (0.6 ml) were collected from a 0 to 350 mM KCl gradient in the presence of buffer A for 90 min at a flow rate of 0.3 ml per minute. Buffer B was used for eluting the peptides, and contained 10 mM potassium phosphate buffer, 350 mM KCl, pH 2.85, 25% acetonitrile. Absorbance at 214 nm was used to monitor the

eluent. Samples were dried using a SpeedVac and desalted using Sep-Pak C18 cartridges (Waters, <http://www.waters.com>) prior to LC-MALDI analysis.

Nano-LC separation and MALDI target spotting of tryptic peptides

Peptide separation was performed on an Ultimate chromatography system equipped with a Probot MALDI spotting device (Dionex, <http://www.dionex.com>). Samples (5 µl) were injected using a Famos autosampler (Dionex) and loaded directly onto a 75 µm × 150 mm reversed-phase column (C18 PepMap 100, 3 µm particles with 100 Å pores; Dionex). Peptides were eluted at a flow rate of 300 nl/min using the following gradient: 0–10 min, 0% solvent B; 10–105 min, 0–50% solvent B; 105–115 min, 50–100% solvent B. Solvent A contained 0.1% TFA in 95:5 water/acetonitrile, and solvent B contained 0.1% TFA in 20:80 water/acetonitrile. For MALDI analysis, the column effluent was directly mixed with MALDI matrix (3 mg/ml α-cyano-4-hydroxycinnamic acid in 70% acetonitrile/0.1% TFA) at a flow rate of 1.1 µl/min. Fractions were automatically deposited every 10 sec onto a MALDI target plate (Applied Biosystems) using a Probot micro-fraction collector. A total of 416 spots were collected from each HPLC run.

MALDI-TOF/TOF mass spectrometry

MALDI plates were analyzed on a 4800 MALDI TOF/TOF system (Applied Biosystems) equipped with a Nd:YAG laser operating at 200 Hz. All mass spectra were recorded in positive reflector mode and generated by accumulating data from 800 laser shots. First MS spectra were recorded from peptide standards, and the default calibration parameters were updated. Then MS spectra were recorded for all sample spots on the MALDI target plate (416 spots per sample, four samples per plate). The MS spectra were recalibrated internally based on the ion signal of neurotensin peptide (Sigma, <http://www.sigmaaldrich.com/>).

Spectral peaks meeting the threshold criteria were included in the acquisition list for the MS/MS spectra. The following threshold criteria and settings were used: mass range = 800–4000 Da; minimum signal-to-noise ratio for MS/MS acquisition = 100; maximum number of peaks per spot = 8. Peptide collision-induced dissociation was performed at a collision energy of 1 kV and a collision gas pressure of approximately 3.3×10^{-4} Pa. During MS/MS data acquisition, a minimum of 1000 shots (20 sub-spectra accumulated from 50 laser shots each) and a maximum of 2000 shots (40 sub-spectra) were allowed for each spectrum. Accumulation of additional laser shots was halted whenever at least six ion signals with a signal-to-noise ratio of at least 60 were present in the accumulated MS/MS spectrum in the region above m/z 200.

Peptide and protein identification by database searching

GLOBAL PROTEOME SERVER EXPLORER software (Applied Biosystems) was used for submitting data acquired with the MALDI-TOF/TOF mass spectrometer for database searching. The MS and MS/MS data were searched using MASCOT version 2.1.0 (Matrix Science, <http://www.matrixscience.com>). All searches were performed against a *Viridiplantae* database (released in April 2008).

The following search settings were used: maximum missed cleavages = 1; maximum number of signals per spectrum = 55; peptide mass tolerance = 25 ppm; MS/MS tolerance = 0.2–0.3 Da. ITRAQ labeling of lysine and the N-terminal amino group of peptides and methyl methanthiosulfonate derivatization of cysteine were specified as fixed modifications. Each peptide also had to be

identified as the top-ranked matching sequence for that spectrum as described by Perkins *et al.* (1999).

Analysis of the iTRAQ data

A meaningful cut-off value for the regulated proteins was established by using the experimental replicate method (Gan *et al.*, 2007; Glen *et al.*, 2008). Data from this preliminary experiment are presented in Figures S3 and S4. A threshold of twofold was found to be stringent.

Following analysis of each reaction using the GLOBAL PROTEOME SERVER EXPLORER software, identified peptides from the control sample (0 h PPD) were selected as the denominator for calculating the fold change of each protein. The ratios for all the peptides were log-transformed to obtain a symmetrical distribution. A combined forward and reverse sequence database (*Viridiplantae*) was searched, and the rate of false-positive scores was calculated according to the method described by Käll *et al.* (2008).

Arabidopsis ortholog mapping and MAPMAN analysis

The identified proteins were mapped to putative orthologs in *Arabidopsis thaliana* complete genome from NCBI (NC_003070.5; GI: 42592260; 4 November 2005) by aligning each protein against all Arabidopsis amino acid sequences (Smith–Waterman significance threshold of $1e-20$) (Smith and Waterman, 1981). If there was more than one significant alignment, the highest-scoring match was selected. The highest-scoring sequence in Arabidopsis is likely to be orthologous, although this is not guaranteed (Dessimoz *et al.*, 2006). Arabidopsis ortholog annotations were used to submit the data to the MAPMAN software (Usadel *et al.*, 2009). Expression patterns, based on log₂ ratios with 0 h as reference, were categorized using the k-means clustering option (with 10 clusters) in MAPMAN.

Clustering

The identified proteins were clustered in groups of homologs using the following greedy bottom-up procedure. A single cluster containing the first match (protein) was initiated, and, for each subsequent match, searches for a high-confidence homolog amongst the already processed matches were performed sequentially. For the purpose of this analysis, homologs were defined as a pair of sequences if their Smith–Waterman alignment (Smith and Waterman, 1981) e-value score was equal to or below $1e-20$. If such a homolog could be found, the match was added to the cluster containing similar homologs, otherwise a new cluster containing this new match was created. Due to the greedy nature of this procedure, the clustering is potentially sensitive to permutations in match ordering. To assess this effect, the procedure was repeated on random permutations of matches. This barely affected the number of clusters and their composition, which indicates that any bias introduced by match ordering is negligible.

Enzymatic assays

Ascorbate peroxidase assay. Ascorbate peroxidase was assayed based on the decrease in absorbance at 290 nm (absorbance coefficient of $2.8 \text{ mm}^{-1} \text{ cm}^{-1}$) as described by Nakano and Asada (1981). The reaction mixture for the ascorbate peroxidase assay contained 50 mM potassium phosphate, pH 7.0, 0.5 mM ascorbate, 0.1 mM hydrogen peroxide, 0.1 mM EDTA and 20 µl of protein extract in a total volume of 1 ml.

Superoxide dismutase assay. Superoxide dismutase (SOD) activity was determined spectrophotometrically in an indirect assay

based on xanthine oxidase and a color reagent using an SOD assay kit (Sigma) according to manufacturer's instructions.

Pectin methyl esterase assay. Pectin methyl esterase (PME) activity leads to acidification of the medium in the presence of esterified pectin. The acidification was monitored using the pH indicator methyl red based on a protocol adapted from that described by Hagerman and Austin (1986).

β -1,3-glucanase assay. Glucanase activity was assayed by the reducing-sugar method (Miller, 1959) using laminarin (*Laminaria digitata*, Sigma) as the substrate. Soluble protein extracts were incubated with 1 mg/ml laminarin dissolved in 50 mM potassium acetate buffer, pH 5, at 40°C for 30 min.

ACKNOWLEDGEMENTS

This work was partially funded by a grant from the Bill & Melinda Gates Foundation (BioCassava Plus program) to W.G. J.A.O. received a PhD fellowship from the Rockefeller Foundation. We thank Evans Nyaboga (ETH Zürich, Switzerland) for comments on the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Reproducibility of the identified proteins between the replicate experiments.

Figure S2. Protein expression profiles of cassava roots during PPD.

Figure S3. Cut-off estimation for the regulated proteins in non-soluble protein fractions.

Figure S4. Cut-off estimation for the regulated proteins in soluble protein fractions.

Table S1. Proteins identified with confidence interval (CI) >95%.

Table S2. False discovery rate estimation.

Table S3. Corresponding Arabidopsis orthologs of identified proteins.

Table S4. Proteins regulated during early and late PPD.

Table S5. Protein clusters.

Table S6. Global overview of the regulated proteins.

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