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Towards identifying the full set of genes involved in post-harvest physiological deterioration in cassava

Reilly K.[1], Diego Cortés[2], Rocío Gómez-Vásquez[1], Tohme J.[2], Beeching J.[1]
[1]Department of Biology & Biochemistry, University of Bath, Bath, BA2 7AY, U.K.
[2]International Centre for Tropical Agriculture (CIAT), AA 6713, Cali, Colombia

Abstract. Cassava is the world's sixth most important crop in terms of production and is a vital staple food to over 500 million in the humid tropics. Unfortunately, it suffers from a rapid post-harvest physiological deterioration (PPD) that can render the roots uneatable and unmarketable within 24-72 hours of harvest. Increased urbanization has lengthened the distance and time between farmers' fields and markets and processors, thereby incurring losses, wastage and discounting of poor quality cassava. PPD is a major constraint to the development of cassava for farmers, processors and consumers alike, and the successful application of strategic research is necessary to solve this problem. With a view to fully understanding PPD and to ultimately producing the tools to control this problem, we have identified some of the key genes that play major roles during PPD and mapped these on the genetic map of cassava. Recently, we have embarked on a major programme employing massively parallel methods of gene analysis (cDNA microarrays) to identify the full set of genes involved in PPD. Screening 11,136 cDNA clones from early and late PPD-related libraries using a series of character probes from a range of time points during the time course of deterioration has led to the identification of 114 clones whose expression is increased and 70 clones whose expression is decreased at least two-fold during PPD.

Introduction

Cassava (*Manihot esculenta* Crantz) is a starchy root crop whose centre of origin is on the south-western borders of the Amazonian basin but which is now cultivated extensively throughout the humid tropics where it provides the staple food for over 600 million (Cock, 1985). Upon harvesting the roots suffer from a rapid deterioration that can render them unpalatable and unmarketable within 24 – 72 hours, known as post-harvest physiological deterioration (PPD). PPD is a major constraint to the development of the crop from a village-based resource to a commodity also supplying urban populations and larger scale processing facilities (Wenham, 1995). The increased times and distances between field and consumer or processor lead to substantial discounting, wastage and added costs due to the effects of PPD. Urban consumers may turn to alternative, often imported, sources of starchy food, processors suffer from unreliable input material and quality control problems, and farmers suffer from loss of potential income. Therefore, research directed towards introducing resistance to PPD, or delaying the response, is considered a priority by international bodies such as the Food and Agriculture Organisation of the United Nations (FAO) and the Cassava Biotechnology Network.
PPD

is an abiotic response of the cassava root to the damage caused during the harvesting process and is not due to microbial action, though this does take place subsequently (reviewed in Beeching *et al*., 1998). Visual symptoms of PPD in the storage root are blue-black discoloration of the vascular tissue, general blue fluorescence under UV light and blockage of the xylem by occlusions and tyloses that can be seen under the microscope. These changes are accompanied by an increase in respiration, the accumulation of a range of secondary metabolites, changes in enzyme activity, protein synthesis and gene expression. These data show that PPD is an active response of the root to harvesting with strong parallels to wound, oxidative stress and senescence responses. Genetic, biochemical and molecular aspects of the problem are now much better understood; for example, several important genes have been cloned and characterised, and the expression of one of these has been analysed in transgenic cassava (Beeching *et al*., 1998; Beeching *et al*., 2002).

Due to its high heterozygosity, a correlation between high dry matter content and PPD, and a complex genetic X environmental interaction, the conventional breeding of cassava for a reduced PPD response is problematic. However, biotechnology can be used to generate an in-depth understanding of PPD, provide molecular markers that could be used for marker assisted selection (MAS) in breeding programmes (Cortés *et al*., 2002), and provide genetic tools to directly manipulate PPD. We have initiated a collaborative programme of research between the University of Bath, U.K., and CIAT, Colombia, to exploit massively parallel systems for the analysis of gene expression in order to identify the full set of genes that are involved in the PPD response. These data should provide valuable insights into the classes of genes involved in the response, and some of these genes themselves together with their promoters could provide gene tools with the potential of modulating the PPD response. While this research programme is only at its halfway point, it has so far proved highly successful at identifying candidate genes. Here we present results on the cloning and screening process, and on the preliminary identification of candidate genes.

Materials and Methods

Plant material. Cassava cultivar CM 2177-2 was grown in the field at CIAT and roots were carefully harvested after about nine months growth.

Induction of PPD. Immediately after harvest proximal and distal ends of the storage roots were removed and two transverse V shaped cuts made along the length of the root. Exposed proximal and distal ends of the root were covered with Parafilm and roots were stored at ambient temp erature. In our hands this system gives an even progression of PPD symptoms throughout the root.

Nucleic acid methods. For cDNA cloning, total RNA was extracted from storage roots according to Reilly *et al*. (2001), and messenger RNA (mRNA) purified with the PolyATtract system (Promega) according to the manufacturer's instruction. mRNA was used to synthesise cDNA and cloned into Lambda Zap II using Stratagene kits according to the manufacturer's instructions. Preparation of cDNA clones, microarray spotting and hybridisations were carried out according to the protocols on the Institute for Genome Research (TIGR) website (http://www.tigr.org/tdb/microarray/). cDNA clones were PCR amplified using T3 and T7 primers and the DNA spotted on to PL-100C-Poly L Lycine slides (CEL Associates) using a Hitachi SPBIO 1.55 robot. Each slide contained four technical replicates of the cDNA clones and four replicates of control genes and target spike genes. cDNA probes were synthesised from mRNA using a SMART cDNA synthesis kit (Clontech). For all experiments the earliest time point cDNA was labelled with Cy3 and the later with Cy5, thus up-regulated genes

appear red. Spike genes used were TIGR E1 – E5 (should appear yellow when probed with Cy3 plus Cy5), TIGR E6 and E7 (should appear green with Cy3 only) and TIGR E8 and E9 (should appear red with Cy5 only). Scanning of the hybridised slides was performed using a VersArray Chipreader (Biorad) and analysis of the hybridisations with ArrayVision (Amersham Biosciences). DNA sequencing was with an Applied Biosystems 3700 DNA Analyzer and 9600 Gold Block Thermal Cyclers. DNA preparation and sequencing reactions were prepared with MWG Biotech Roboprep 2500 and Roboseq 4200 robotic systems. All sequencing reactions were with Applied Biosystems' Big-Dye Terminators. BLAST searches to tentatively identify clones were carried out on the NCBI website (http://www.ncbi.nlm.nih.gov/) (Altschul *et al*., 1997). Multiple alignments were made using CLUSTAL (Higgins *et al*., 1996). All other molecular methods were standard (Sambrook, *et al*., 1989).

Results

cDNA library construction and evaluation. Messenger RNA was purified from mature roots of cassava cultivar CM 2177-2 over a post-harvest time course at 0, 6, 12, 24, 48 and 96 hours after harvesting. mRNA from time points 0, 6 and 12, and from 24, 48 and 96 hours was pooled separately and used to synthesise cDNA, which was directionally cloned into Lambda Zap II; thereby creating an "Early PPD" and a "Late PPD" cDNA libraries. These libraries were evaluated as to their size and quality. The "Early PPD" library contained $1.4x10^7$ plaque-forming units (pfu) per ml, of which 11.2% were non-recombinants and at least 80% of the insert sizes were greater or equal to 0.9 kilobase pairs (kb) in size, and the "Late PPD" library contained $3.8x10^9$ pfu/ml, of which 0.8% were non-recombinants and 70% were greater or equal to 0.9 kb.

cDNA microarray hybridisations. DNA PCR amplified from 7,680 clones of the "Early PPD" library and 3,456 of the "Late PPD" library, a combined total of 11,136 clones, were spotted onto slides. Each slide contained four technical replicates of each clone together with four replicates of the control genes and target spike genes. These slides were hybridised with Cy3 and Cy5 labelled probe cDNA as in Table 1, the earlier probe was always labelled with Cy3 and the later one with Cy5; thus up-regulated clones should appear red and down-regulated green. These hybridisations proved highly successful.

Microarray data analysis. The hybridised slides were scanned using the VersArray Chipreader and the data analysed using ArrayVision software. Two criteria were used to identify clones for further analysis: one in which clones were flagged for each hybridisation, e.g. 0 vs. 12, 0 vs. 24 hours, etc.; and one involving analysis of data normalised across arrays. Only clones that showed up-regulation by at least a factor of two (2^1) or a down-regulation of at least 2.8 ($2^{1.5}$) in at least two array hybridisations were selected. The higher factor for the down-regulated clones was used due to the large number of clones that were down-regulated (green) in order to reduce these to a manageable number. These strict criteria should eliminate most false positives and not too many real positives. Using these methods,

Table 1: Outline of the microarray hybridisation experiments.

Experiment	Hybridisation	Probes
Time course experiments	Hybridisation 1	0 vs. 12 hours
	Hybridisation 2	0 vs. 24 hours
	Hybridisation 3	0 vs. 48 hours
	Hybridisation 4	0 vs. 72 hours
	Hybridisation 5	0 vs. 96 hours
Range experiments	Hybridisation 6	12 vs. 24 hours
	Hybridisation 7	24 vs. 48 hours
	Hybridisation 8	48 vs. 72 hours
	Hybridisation 9	72 vs. 96 hours

Hybridisations were carried out with Cy3 (the earlier time point) and Cy5 (the later time point) labelled cDNA probes to determine the time course and range of changes in gene expression in 11,136 PPD-related cDNA clones.

out of the total of 11,136 cDNA clones examined, 114 clones showing at least a two-fold increase in gene expression during PPD were identified together with 70 clones showing at least a 2.8-fold decrease in expression.

Candidate clone analyses. The 184 clones identified above were retrieved from frozen stocks and plasmid DNA purified for single pass sequencing in the forward direction using the T3 primer in order to generate expressed sequence tags (ESTs) for these clones. These sequences were submitted to the NCBI website for BLASTN and BLASTX database searches in order to tentatively identify them by comparison to the DNA and amino acid sequences of other genes. These data and their analyses are as yet preliminary and require further work. However, initial analyses permitted the tentative identification of most of the clones and there placing into major classes. Some of these clones were of particular interest. Amongst the up-regulated clones we will highlight the following. An ascorbate and a guiacol peroxidase which have not been described in cassava so far – peroxidases are known to play important roles in PPD and in a reaction with scopoletin that leads to the blue-black staining of the vascular tissue (Reilly *et al*., In press). A germin-like protein – these have only recently been described in plants and are involved in stress and defence responses. ACC oxidase – this is involved in the synthesis of the phytohormone ethylene and for which we have already got cDNA and genomic clones. 10 cytochrome P450s – these are one of the largest gene families in *Arabidopsis* and have many different functions from lignin and suberin biosynthesis to the synthesis or catabolism of signalling molecules. Multiple sequence alignment of these clones shows that they fall into four discrete types (Figure 1). Two glucosyl transferases, these catalyse the transfer of sugars to a range of acceptor molecules; in plants glucosyl transferases are a large family of genes and their precise roles and substrate specificities are unclear. Phospholipase – this has a possible role in signal transduction. Amongst the down-regulated clones the following look of

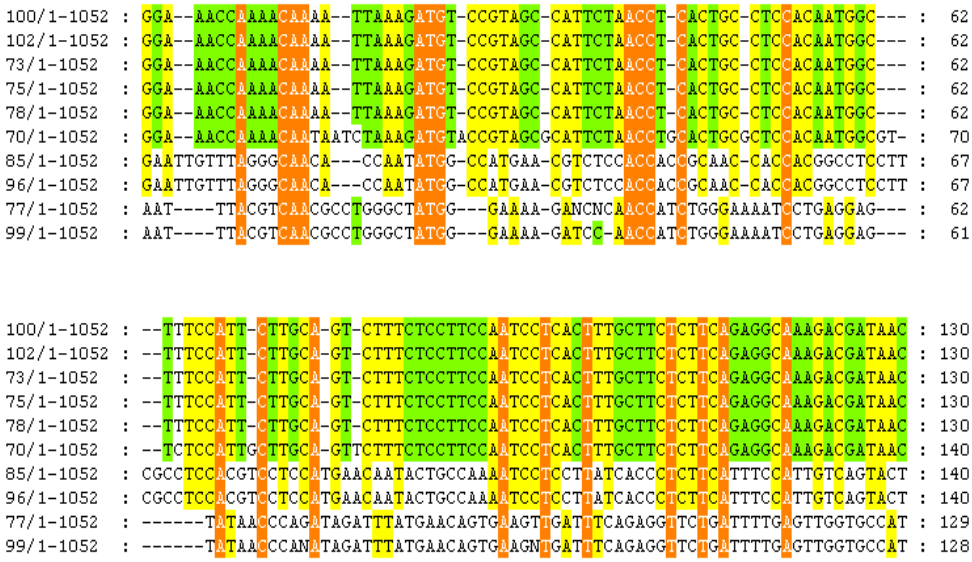

Figure 1: Multiple alignment of cytochrome p450 sequences. The DNA sequences of the 10 cDNA clones tentatively identified as cytochrome P450s were aligned using CLUSTAL. This permitted their assignment to four distinct classes.

particular interest. An auxin-repressed protein – these are often associated with dormancy. An IAA amidohydrolase – these are involved in the regulation of levels of free IAA. ADP ribosylation factor – these are small G-proteins that play a central role in intracellular vesicle transport and some have been shown to regulate phospholipase D activity. A senescence-associated gene – these are involved in programmed cell death.

Discussion

Two PPD-related cDNA libraries were created, one "Early" containing pooled samples from 0, 6 and 12 hours after harvest, and one "Late" from 24, 48 and 96 hours. 11,136 clones from these libraries were hybridised with Cy3 and Cy5 labelled cDNA probes synthesised from mRNA purified from a range of time points over a PPD time course. Four technical replicates of each clone and a selection of control DNAs enabled these hybridisations to be normalised and for clones corresponding to genes whose expression had increased or decreased according to two criteria to be identified. The criteria applied to the down-regulated genes were stricter than those applied to the up, as otherwise an unmanageable number of clones would have been selected. However, it is possible that the criteria used were too strict and that some clones, especially those that show transient changes in gene expression, may have been missed. It is important to point out that these are preliminary data and that it is important to complete a further two sets of biological replicates in order to have full confidence in the results. These biological replicates are currently in progress; when they are ready and their data combined with that discussed here, this will increase the confidence that can be placed in the overall analysis. As a result of which, it may prove possible to relax somewhat the criteria for clone selection, thereby identifying some potentially important clones, particularly those whose expression changes transiently, that have so far been missed. However, database searches using BLAST enabled some of the clones to be tentatively identified and to be assigned to functional classes, these classes included signalling, oxidative stress and defence. Previous work in our laboratories have identified and highlighted the important roles that oxidative stress and the enzymes and compounds that modulate it play in the PPD response of the cassava root (Buschmann *et al*., 2000a; Buschmann *et al*., 2000b; Reilly *et al*., 2001; Reilly *et al*., in press). Therefore, it will be of great interest to fully characterise the clones identified here and to evaluate their role in this stress response. In addition the signal transduction pathways leading to the PPD symptoms involve reactive oxygen species (ROS) at certain steps; while these overall pathways are likely to be complex the signalling components identified here, together with those that we have already characterised promise to add to our understanding of this aspect of the response.

Two other studies of the classes of genes expressed during PPD are available; one using cDNA-AFLPs (Huang *et al*., 2001) and a second using differentially screened and random PPD-related cDNA clones (Beeching, 2001; Gómez-Vásquez *et al*., 2001). Of the 70 transcript derived fragments (TDFs), 40 were similar to known genes; these included TDFs for genes involved in stress responses, metabolism, signal transduction and development (Huang *et al*., 2001). Beeching (2001) characterised 92 PPD-related clones, of which 78 could be tentatively identified based on similarity to known genes; a major class of these were cell wall proteins, though it was possible that the methodology used biased the sampling in this direction (Gómez-Vásquez *et al*., 2001). Other classes included genes involved in the regulation of gene expression, transcription, translation metabolism, signal transduction, stress responses and senescence. It is anticipated that when the current project has advanced further, these studies will complement it and add to the developing picture to the molecular biology of the PPD response.

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