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Hydroxyproline-rich glycoproteins expressed during stress responses in cassava

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Summary

The storage roots of cassava (*Manihot esculenta* Crantz) suffer from a rapid post-harvest deterioration that is a major constraint to their increased exploitation. In many ways this deterioration resembles wound responses in other better studied plant systems, though it appears to lack an adequate wound repair response. A cDNA clone (cMeHRGP1) for a hydroxyproline-rich glycoprotein expressed during the deterioration response was isolated and characterised. This clone proved to be an antisense pairing, coding for part of phosphoserine aminotransferase on its complementary strand. Messenger RNA corresponding to cMeHRGP1 accumulated in deteriorating cassava roots from day three after harvest, by which time the deterioration response was well advanced. Thereby confirming that aspects of the wound repair response were inadequate in harvested cassava roots.

Abbreviations: HRGP – hydroxyproline-rich glycoprotein; PAL – phenylalanine ammonia-lyase; PPD – post-harvest physiological deterioration; PSAT – phosphoserine aminotransferase

Introduction

Cassava (*Manihot esculenta* Crantz) is the staple root crop of over 500 million people in the humid tropics (CIAT, 1992) and ranks sixth in terms of overall global production (Mann, 1997). Its productivity, its resistance to drought, and its ability to grow on marginal soils with the minimum of input make cassava a vitally important crop to some of the world's poorest populations and a significant famine reserve crop (Cock, 1985). However, despite these advantages cassava does suffer from several problems, significant amongst which is a rapid post-harvest deterioration. Within 24–48 hours after harvest, depending of the cultivar, cassava roots undergo changes that make them unpalatable and unmarketable (Beeching et al., 1994). These changes, known as post-harvest physiological deterioration (PPD), necessitate the prompt consumption or processing of the cassava roots after harvesting. Traditionally, PPD has not been a significant problem as cassava has been grown and consumed or processed locally immediately after harvesting, often being kept in the ground until it is required. However, with increased urbanisation and interests in larger scale production and processing, markets are now at greater distances and processing can entail delays. Therefore, PPD has become a major constraint to the use and development of cassava in terms of production, consumption and processing. Estimated losses from PPD range from 5–25% of the harvested roots (Wenham, 1995). In Colombia it has been estimated that annual net losses amount to US$ 5 million for this country alone (C. Iglesias, personal communication). Another direct consequence of PPD in cassava is the high marketing margins for fresh root markets; margins can be as high as 60% of the final retail price (Janssen &

Wheatly, 1985). In addition, urban populations look to more reliable or economic alternatives to cassava for carbohydrate in their diets, which leads to the import of wheat for bread-making, thereby weakening national and rural economies. Therefore, research directed towards delaying PPD has been given a high priority by the Cassava Biotechnology Network and by the FAO (Anonymous, 1994; Wenham, 1995).

Post-harvest deterioration in cassava occurs in two stages: the first in which there are physiological, biochemical and ultra-structural changes in the root and does not involve micro-organisms, and a second subsequent stage in which microbial decay occurs (Rickard & Coursey, 1981; Wheatley & Gomez, 1985). It is the former, known as post-harvest physiological deterioration (PPD), that is the constraint to the development of cassava. PPD is initially observed in transverse sections of the root as a blue-black discoloration of the vascular tissue, known as vascular streaking, which spreads from initial surface wound sites produced by the process of harvesting (Booth, 1976). A strong blue fluorescence under ultra violet light due to the production of phenolic compounds, including scopoletin, precedes the discoloration (Wheatley & Schwabe, 1985). Associated with the discoloration, coloured occlusions and tyloses are formed from the xylem parenchyma that block adjacent xylem vessels (Richard & Gahan, 1983). Biochemically, PPD is accompanied by an increase in respiration and mobilisation of starch to sugars, the activity of acid invertase increases (Tanaka et al., 1983). Production of the phytohormone ethylene increases (Hirose et al., 1984); ethylene is considered to play a role in co-ordinating wound and senescence responses in plants generally (Ecker & Davis, 1987). The activity of various enzymes including dehydrogenases, peroxidases, catalases, phenylalanine ammonia lyase (PAL) and phenol oxidase increase during PPD (Czyhrinciw & Jaffé, 1951; Hirose, 1986; Plumbey et al., 1981). Changes in lipid composition and the increased synthesis of phenolic compounds, diterpenes and catechins occur in the cassava root during PPD (Lalaguna & Agudo, 1989; Sakai & Nakagawa, 1988; Wheatley & Schwabe, 1985). It is probable that the observed discoloration is the product of interactions between some of these secondary metabolites and oxidative enzymes. Evidence from cyclohexamide inhibition, *in vivo* labelling of proteins and cDNA cloning indicates that PPD is an active process involving changes in gene expression and the synthesis of novel proteins (Beeching et al., 1995; Beeching et al., 1997; Uritani et al., 1984).

These features of cassava PPD are common to those observed in wound and stress-induced responses in other better studied plants. This suggests that the differences between PPD and normal plant wounding may be more ones of emphasis than of kind. In addition, it implies that plant wounding can provide useful conceptual and practical tools for the understanding of cassava PPD. A plant wound response can be simplistically broken down into three interrelated components (Bennett & Wallsgrove, 1994; Bowles, 1990). Firstly, signals are produced and act locally to the wound site. These signals are either produced as a direct consequence of wounding, such as membrane peroxidation products or cell-wall fragments, or are induced by the wounding, such as jasmonic acid, abscisic acid, salicylic acid, systemin or H_2O_2. Other signals act systemically and prepare the plant for the extension of wounding or pathogen invasion; these include systemin, electrical and hydraulic signals. Secondly, the plant produces defensive enzymes and molecules that serve roles in the protection of the plant against pathogens or the effects of wounding. These include enzymes such as glucanases and chitinases that attack components of microbial cell walls, and secondary metabolites, including phenolics, that can act as antimicrobials (phytoalexins) or anti-oxidants. Thirdly, wound repair occurs via the synthesis of suberin and lignin from phenolic components, callose synthesis, the insolublisation of hydroxyproline-rich glycoproteins by H_2O_2, and the formation of a wound meristem. This repair leads to the sealing of the wound, the inhibition of the production of the signals triggering the wound response, and a return of the plant to normal development. Branch pathways from general phenylpropanoid metabolism lead into all these aspects of the wound response: the biosynthesis of the signalling molecule salicylic acid, the production of phenolic anti-microbials and anti-oxidants, and the synthesis of components for the biosynthesis of suberin and lignin (Dixon & Paiva, 1995; Hahlbrock & Scheel, 1989).

Recent research at the University of Bath and in collaboration with CIAT has confirmed and extended the conclusion that much of the plant wound response is found in cassava PPD. In terms of signals and signal transduction, an increase in H_2O_2 production has been observed during the early stages of PPD (Reilly et al., 2000), there is expression of the ethylene biosynthetic enzyme 1-aminocyclopropane-1-carboxylate oxidase

(Li et al., 2000), and a putative serine-threonine protein kinase has been cloned from a PPD-related cDNA library (Han et al., 2000). Components of the defensive response have been identified; for example secondary metabolites with anti-microbial and anti-oxidant properties have been characterised (Buschmann et al., 2000; Rodriguez et al., 2000) and a β-1,3-glucanase has been cloned (Han et al., 2000). Three genes for phenylalanine ammonia-lyase (PAL), the key entry enzyme to general phenylpropanoid metabolism, have been cloned, of which two at least are expressed during PPD (Beeching et al., 2000). The exploitation of cassava cell-suspension cultures has also enabled the dissection of the possible range of cassava's stress responses using a highly controlled model system. This approach has shown that, in response to fungal cell-wall elicitors, cassava suspension cells are capable of producing most of the repertoire of enzymes and secondary metabolites observed in the roots during PPD. In addition, these cassava cells are capable of generating reactive oxygen species (ROS) in response to elicitors and an oxidative burst in response to incompatible pathogens (Beeching et al., 1998).

All evidence indicates that the signalling and defensive components of plant wound responses are found in the cassava storage root's PPD response. However, the wound healing aspects appear to be inadequate. In normally harvested cassava roots there is neither deposition of suberin or callose, nor formation of a wound periderm (Booth, 1976). Therefore, there is no cessation of production of the initial signals that trigger the wound-response, leading to a continual cascade of defensive responses that spread through the root and are observed as PPD (Beeching et al., 1994). However, wound repair does occur in roots that remain attached to the plant. In addition, wound repair can be induced in harvested roots that are kept at high temperatures and humidity; under these conditions suberin deposition and periderm formation in the cortex are observed, albeit at lower rates than in other tropical roots such as yam (Booth, 1976). Moreover, PPD can be inhibited by storing roots under conditions from which oxygen is excluded (Rickard & Coursey, 1981), suggesting that oxygen and ROS play a role in the deterioration response.

These results show that cassava roots have the capacity for at least near normal wound repair, but that this response is prevented or inadequate in conventionally harvested and stored roots. Cassava storage roots do not function as propagules as do many tubers or tuberous roots. Their principal function is to serve as a repository of photosynthate for use by the plant to recover from defoliation after periods of drought. For these reasons the storage roots serve no biological function once detached from the plant. Therefore, there are no biological selective pressures for the retention and maintenance of normal wound responses, including repair, in the detached root. Cassava was domesticated in the Amazon basin over 8,000 years ago (Olsen & Schaal, 1999; Piperno & Holst, 1998). These farmers probably used cassava in ways similar to those employed by Amazonian communities today; freshly harvested cassava is either prepared or processed directly for food or drink, or it is promptly processed into a readily transportable flour-like product. Therefore, while we can speculate as to whether or not the storage roots of the wild progenitor of cassava were capable of healing after harvest, during the several thousands of years since its domestication there were probably no human derived selective pressures on it for the retention of healing responses in the detached root.

In this paper we examine a component of the wound healing response that is present in harvested cassava storage roots undergoing PPD, the characterisation of a hydroxyproline-rich glycoprotein expressed during the deterioration response.

Materials and methods

Plant material

Cassava plants (cultivar MDOM 5) were grown in the field at CIAT, Cali, Colombia.

Cassava cell suspension cultures (cultivar MCOL 22) were grown on liquid MS medium (Murashige & Skoog, 1962) supplemented with 2% sucrose and 2 mgl^{-1} 2,4-dichlorophenoxyacetic acid. The cultures were maintained by shaking in an orbital incubator in the dark at 110 rpm at 25 °C, and sub-cultured every seven days.

Yeast cell-wall elicitation experiments

Glucan cell-wall elicitor was prepared from baker's yeast (Schumacher et al., 1987). Cassava cell suspension cultures were used five days after subculture. Yeast elicitor was added at a concentration of 50 μg/ml of glucose equivalent to 50 ml cell cultures. Samples of the cells were taken over a time course after elicitation by harvesting onto a porous glass

filter (Whatman No 1), washing twice with sterile deionised water, frozen in liquid nitrogen and stored at –80 °C until used.

Conditions for deterioration experiments

Cassava storage roots (cultivar MDOM 5) were carefully harvested from field-grown plants at CIAT, the proximal and distal ends were removed, the roots stored in the shade at ambient conditions, and samples taken at daily intervals for RNA extraction.

DNA methods

A cDNA library was constructed in λgt10 from poly(A)$^+$ RNA isolated from 48 hour harvested storage roots from cassava cultivar MNGA 1 (Beeching et al., 1997). Cassava genomic DNA was isolated from young leaves (Dellaporta et al., 1983). DNA sequencing was on an Applied Biosystems ABI 337 automated sequencer. All other DNA methods were standard (Sambrook et al., 1989). PCR primers, whose products are discussed in this paper, were: h1rf (ACGGAGATACAGCCTCGGCCTTCTG, from bases 281–304 of cMeHRGP1), xp1 (CCTCCTCCTCCATACTACTAT, from bases 456–476), xf1 (TTACGCCTCACCATAGGCTC, from bases 110–1119), and xr1 (GTCCAAGAAAGAATCGCTCAC, from bases 1539–1519).

RNA methods

Total RNA was extracted from 0.5g cassava suspension cells using the SV total RNA isolation kit (Promega). From storage roots, total RNA was extracted using the method of Chang et al. (1993). Total RNA (10 μg) was electrophoresed on a 1% agarose gel containing formaldehyde and blotted onto nylon membrane (Hybond N+, Amersham) according to standard procedures (Sambrook et al., 1989). Northern hybridisations were carried out with α^{32}P-labeled cDNA probes at 65 °C overnight in 0.5M Phosphate buffer (pH 7.2), 1% skimmed milk, 7% SDS. Membranes were washed in 1 × SSC, 0.1% SDS at room temperature for 10 minutes, three times in 0.2 × SSC, 0.2% SDS for 20 minutes at 65 °C and then visualized by autoradiography at –70 °C.

```
        MAGPAAKLKT SP
SPPPP YYYK SPPPP SP
SPPPP YYYK SPPPP SP
SPPPP YYYH SPPPP VK
SPPPP YYYH SPPPP VK
SPPPP YYYH SPPPP VK
SPPPP YYYH SPPPP VK
SPPPP YYYH SPPPP VK
SPPPP YYYH SPPPP VK
SPPPP YYYH SPPPP VK
SPPPP YYYH SPPPP VK
SPPPP YYYH SPPPP VK
SPPPP YYYH SPPPP VK
SPPPP YYYH SPPPP VK
SPPPP VYIY ASP
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Figure 2. The deduced amino acid sequence of cMeHRGP1 is arranged in order to display the various amino acid repeat units and their periodicities. S-serine, P-proline, Y-tyrosine, K-lysine, H-histidine, V-valine, I-isoleucine, T-threonine, A-alanine, L-leucine and G-glycine.

Results

Isolation of a cDNA clone for a hydroxyproline-rich glycoprotein

The PPD-related λgt10 cDNA library was screened with parsley HRGP cDNA ELI9 as a probe (a generous gift of I.E. Somssich (Kawalleck et al., 1995)) at a stringency of 0.1 × SSC at 50 °C; nine positive clones were identified. Preliminary PCR screening of these clones showed that their insert sizes ranged from approximately 100 bp to 1.9 kb (data not shown). The insert of one of the larger clones was sub-cloned into the *Eco*RI site of pUC18 and its DNA sequence determined using universal and specifically designed primers (Figure 1). The cDNA was 1649bp in size, with a 416bp 5′ untranslated region, 669bp coding region, a 494bp 3′ untranslated region and a poly(A) tail. The initiation ATG was identified by searching for the first ATG in the longest open reading frame, and by examining the context in which it was found. The ATG at nucleotide 417 was found within the sequence ATAAGATGGCA which is very close to the consensus, aaA(A/C)aATGGCu, for dicotyledonous plants (Joshi et al., 1997). A putative polyadenylation site (AATAAA) was located 28bp upstream of the poly(A) tail.

The coding sequence showed 79% identity to the HRGP genes of soybean (Genebank accession

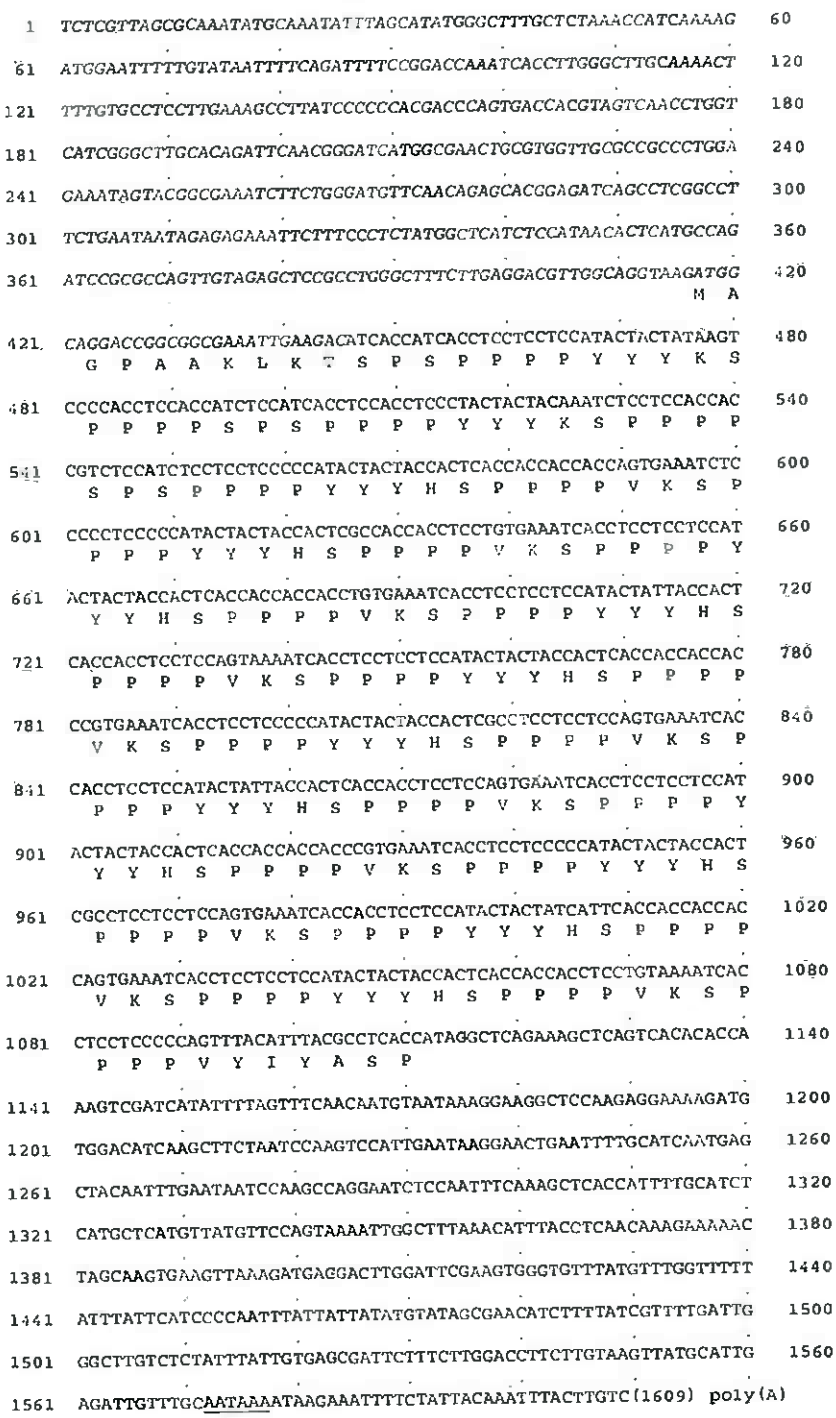

Figure 1. The nucleotide and deduced amino acid sequences cMeHRGP1. The translation start and stop codons of HRGP1 are in bold. The putative polyadenylation addition signal is underlined. The region that codes for part of phosphoserine aminotransferase (PSAT) on the antisense strand is in italics.

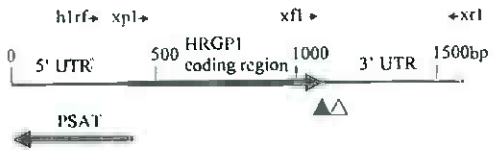

Figure 3. Diagram of clone cMeHRGP1 showing positions of the coding region for HRGP1, 5′ untranslated region (UTR) and 3′ UTR. The positions of PCR primers h1rf, xp1, xf1 and xr1 are indicated. The region of cMeHRGP1 that codes for part of phosphoserine aminotransferase (PSAT) on the antisense strand is shown. The white triangle indicates the position of the 106bp intron in the genomic equivalent of cMeHRGP1. The black triangle indicates the position of the extra six codons in cMeHRGP2.

number L22031) and bean (M18095) over 500bp (data not shown). The cDNA clone was designated cMeHRGP1; its sequence has been deposited with GenBank with the accession number AF239615. The deduced amino acid sequence of the coding region contained repeat motifs such as SP_4, a typical repeat unit of HRGPs (Figure 2). There were two SP_4-Y_3K-SP_4-SP motifs, eleven SP_4-Y_3H-SP_4-VK repeat units and one SP_4 near the C- terminal in the deduced amino acid sequences. The deduced peptide contained 27 $S(P)_4$ repeats in total. The amino acid composition (mole%) of the predicted polypeptide was 48.71% proline, 17.67% tyrosine, 13.36% serine, 6.47% lysine, 5.17% valine, 4.74% histidine and 3.88% other amino acids.

cMeHRGP1 shows antisense gene pairing

A search of the DNA database at NCBI (http://www.ncbi.nlm.nih.gov/) using the Blastx program (Altschul et al., 1997) with the cMeHRGP1 sequence revealed that the deduced amino acid sequence derived from the first 445bp of the complementary strand of the clone was similar to that of phosphoserine aminotransferase (PSAT) from *Arabidopsis thaliana* (GenBank accession number G88541) (Figures 1 and 3). Phosphoserine aminotransferase catalyses the conversion of phosphohydroxypyruvate to phosphoserine in the phosphorylated pathway of serine biosynthesis. This finding, coupled with the observation that cMeHRGP1 has unusually long 5′ and 3′ untranslated regions, raises the question as to whether this clone was a chimerical hybrid produced by the cloning process, or whether it was a genuine cDNA clone derived from a region of the cassava genome with the HRGP1 gene on the sense strand and possessing a PSAT gene or gene-derived sequence on the antisense strand, a so called antisense gene pairing.

In order to answer this question pairs of PCR primers were chosen which spanned critical regions of the clone; these were used to amplify cassava genomic DNA. PCR of genomic DNA using primers h1rf and xr1 (Figure 3) amplified a product that was marginally larger than the corresponding control product from cMeHRGP1 (data not shown). The genomic DNA derived product was partially sequenced from both ends using the same primers. The sequence from the 5′ end of the product using primer h1rf provided readable data of approximately 500bp. While this sequence data was not perfect, due to the ambiguities often encountered when sequencing a PCR product, it showed over 80% similarity to cMeHRGP1 (data not shown). The sequence spanned the junction between the phosphoserine aminotransferase (PSAT) and the HRGP coding sequence from nucleotides 350–812 of cMeHRGP1.

The 3′ sequence data from this PCR product using primer xr1 was of much higher quality than that obtained with h1rf. This sequence was identical to the 3′ end of cMeHRGP1 except for the presence of 18 extra nucleotides immediately preceding the stop codon, which encoded an additional six amino acids, thereby adding another SP_4 to the polypeptide (Figure 4). This suggested that this amplification product was not from the gene corresponding to cMeHRGP1, but from a very closely related family member that differed from cMeHRGP1 by the presence of these six extra codons. This was confirmed by sequencing another of the nine cDNA clones isolated from the library, which proved to be an incomplete HRGP clone with the same six extra codons preceding the stop codon. This clone was called cMeHRGP2. While the PCR sequencing data did not categorically prove that cMeHRGP1 was not a hybrid clone, it did prove that cMeHRGP1 had a very close family member, cMeHRGP2, that was an antisense gene pair. The implication being that cMeHRGP1 was so too.

Additional evidence that cMeHRGP1 was not a hybrid clone comes from sequence analysis of a genomic PCR product generated using primers spanning the unusually long 3′ end of the clone. Primers xf1 and xr1 were designed from near the end of the coding region and near the poly(A) tail of cMeHRGP1, respectively (Figure 3). Primer xf1 included the last four codons, including the stop codon, and the first four nucleotides of the 3′ untranslated region of cMeHRGP1, and so should amply the corresponding genomic sequence of cMeHRGP1 and not that of cMeHRGP2. These primers amplified a PCR product from cas-

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cMeHRGP1  1092 GTTTACATTTACGCCTCACCA.....................TAGGCTCAGAA 1123
               ||||||||||||||||||||                     ||||||||||||
PCR sequence   GTTTACATTTACGCCTCACCACCACCACCAACTCACTACTAGGCTCAGAA
               V  Y  I  Y  A  S  P  P  P  P  T  H  Y  *
```

Figure 4. Alignment of a part of the cMeHRGP1 sequence, corresponding to the carboxy terminus of the protein, with part of the 3 end of the genomic sequence amplified with primers h1rf and xr1. The 18 extra nucleotides in the PCR product and the corresponding six amino acids are in italics. The stop codon in both sequences is in bold.

```
   1  GCCTCACCATAGGCTCAGAAAGCTCAGTCACACACCAAAGTCGATCATgtaagtttcttt   60
      A  S  P

  61  aacacttctaacatcatttttcaagttttttagtattagcaaaatgaatcattttgaagt  120

 121  gtatgtctaattttctgccatttccatacaacagATTTTAGTTTCAACAATGTAATAAAG  180

 181  GAAGGCTCCAAGAGGAAAAGATGTGGACATCAAGCTTCTAATCCAAGTCCATTGAATAAG  240

 241  GAACTGAATTTTGCATCAATGAGCTACAATTTGAATAATCCAAGCCAGGAATCTCCAATT  300

 301  TCAAAGCTCACCATTTTGCATCTCATGNTCATGTTATGTTCCAGTAAAATTGGCTTTAAA  360

 361  CATTTACCTCAACAAAGAAAAACTAGCAAGTGAAGTTAAAGATGAGGACTTGGATTCGAA  420

 421  GTGGGTGTTTATGTTTGGTTTTTATTTGTTCATCCCCAATTTATTATTATATGTATAGCG  480

 481  TACATCTTTTATCGTATTGATTGGCTTTTCTATATTTATT    520
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Figure 5. Sequence of the genomic PCR product corresponding to the 3′ untranslated region of cMeHRGP1. The nucleotides in capital letters are shared sequences between cMeHRGP1 and the PCR product, the stop codon is in bold. The intron is in lower case and the splice sites are in bold.

sava genomic DNA that was about 100bp larger than that amplified from cMeHRGP1 control DNA. Sequencing this genomic PCR product showed that it had the same carboxy terminal codons as cMeHRGP1 and that it contained a 106bp intron in a sequence that was otherwise identical to the 3′ untranslated region of cMeHRGP1 (Figure 5). The intron possessed the GT and AG splice sites that are common to plant introns. Interestingly, the sequence of the genomic PCR product generated with primers h1rf and xr1, discussed above, showed that this intron was not present in the genomic sequence corresponding to the 3′ untranslated region of cMeHRGP2.

Cassava HRGP gene organisation

Southern blot analysis was performed on cassava genomic DNA in order to estimate the size of the HRGP gene family. The probe used was a PCR product from cMeHRGP1 generated with primers xp1 and xr1 that spanned the coding region of the HRGP and the 3′ untranslated region (Figure 3). Restriction enzymes were chosen that did not cut cMeHRGP1 within the region covered by the probe. The Southern blot was washed at low and at high stringencies in order to detect distant and close family members, respectively (Figure 6). At low stringency multiple hybridising bands were present in all three digests, indicating that the cassava genome contained several genes or sequences with 82% or more similarity to the cMeHRGP1 probe. When the membrane was washed at higher stringency (equivalent to 93% similarity), a single prominent band of large size was present in the *Hin*dIII and *Bgl*II lanes. However, two smaller bands of nearly the same size were just distinguishable from each other in the *Xba*I lanes upon a close inspection of the original autoradiograph. These results show that in cassava, HRGPs are encoded by a multigene family including members with a range of degrees of similarity, and that within this family there are two members with very high similarity to cMeHRGP1, probably the genes corresponding to cMeHRGP1 and cMeHRGP2. Partial sequencing of the other cDNA clones indicated that these were also for HRGPs, though none of

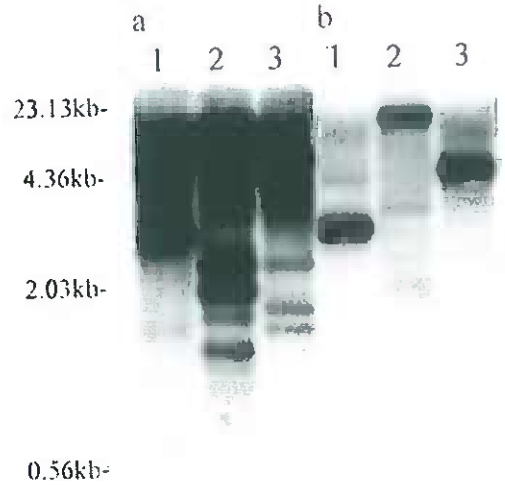

Figure 6. HRGP gene organisation in cassava. Southern hybridisation of restriction digests of cassava genomic DNA (10 μg) probed with a cMeHRGP1 derived PCR product. 1 = *Xba*I, 2 = *Hin*dIII, and 3 = *Bgl*II. a: Low stringency (60 °C, 1 × SSC). b: High stringency (60 °C, 0.1 × SSC). In the original autoradiograph two separate bands of nearly the same size were just distinguishable in the *Xba*I lane of the high stringency hybridisation.

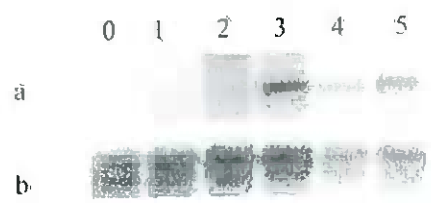

Figure 7. Expression of HRGP gene in cassava roots over a time course of five days after harvesting. Northern blots of 10 μg total RNA probed with: **a** - cMeHRGP1; **b** – probed with cassava 18S rRNA gene, as a control for equal RNA loading. High stringency wash (68 °C, 0.2 × SSC).

them contained the complete coding sequence. One of which (cMeHRGP3) contained WP_4 and HP_3 repeats in addition to the SP_4 repeats found in the deduced amino acid sequence of cMeHRGP1 (data not shown). This confirmed that the cassava HRGP gene-family contains diverse members.

Expression of HRGP genes during post-harvest deterioration and in response to elicitor

The storage roots of MDOM 5, used for these experiments, showed initial visible signs of post-harvest

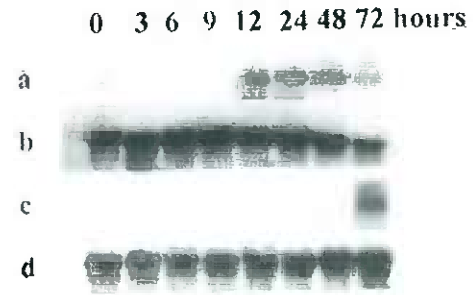

Figure 8. Northern blots showing expression of HRGP gene in cassava cell suspension cultures challenged with yeast elicitor. a – Time course of HRGP mRNA accumulation after addition of yeast elicitor, probed with cMeHRGP1. **b** – The same blot as a, probed with a cassava 18S rRNA gene as control for equal loading. c – Time course of HRGP mRNA accumulation in unchallenged cells, probed with cMeHRGP1. **d** – The same blot as c, probed with cassava 18S rRNA gene as control for equal RNA loading. Ten μg total RNA per lane, high stringency washes (68 °C, 0.2 × SSC).

physiological deterioration (PPD) within 24 hours of harvest. However, mRNA corresponding to the cMeHRGP1 probe was not detectable during the first two days of a deterioration time-course on Northern blots of total RNA probed with cMeHRGP1 (Figure 7). A strong HRGP1 signal was detected on day three, which reduced slightly in intensity over the next two days. By day three the PPD symptoms in the root were very marked. Therefore, the symptoms of PPD precede the accumulation of HRGP1 mRNA. In cassava cell-suspension cultures, glucan cell wall elicitor isolated from baker's yeast induced a rapid change in HRGP1 gene expression, with an accumulation of messenger RNA 12 hours after elicitation (Figure 8). This contrasted with the non-elicited cells, in which HRG1 mRNA accumulation was only detected after 72 hours. Estimations of the size of the mRNA detected on the Northern showed that it was over 4000 bases; this is considerably larger than the 1649bp of cMeHRGP1. This implies that the full length transcript of the corresponding gene is at least twice the size of the cDNA sequenced here.

Discussion

Nine HRGP clones were isolated from a cassava PPD-related cDNA library, three of which, cMeHRGP1, cMeHRGP2 and cMeHRGP3, fell into two different classes. cMeHRGP1 and cMeHRGP2 were virtually

identical except that the latter encoded six extra amino acids at its carboxy terminus, and the gene corresponding to the former had a small intron of 106bp in its 3′ untranslated region. Introns in 3′ untranslated regions also occur in HRGP genes of other plants (Chen & Varner, 1985). cMeHRGP3, though it contained the SP_4 repeats found in the other two, contained WP_4 and HP_3 repeats in addition. Southern hybridisation confirmed that the cassava genome contained an HRGP gene-family with varying degrees of similarity. Only cMeHRGP1 was a full-length clone, so it alone was analysed in detail. This clone (and cMeHRGP2), which had unusually long 5′ and 3′ untranslated regions, proved to be an antisense pairing, coding for part of phosphoserine aminotransferase (PSAT) in its complementary strand. Sequencing genomic PCR products dismissed doubts that this was a cloning artefact, and confirmed that there was a genomic counterpart.

Long reading frames on the antisense strand of open reading frames are more frequent than expected (Silke, 1997). The antisense of many genes may code for unidentified proteins (Knee & Murphy, 1997; Silke, 1997). Deduced amino acid sequences of antisense strands in some yeast genes showed high homology to sequences in the Genebank database (Cebrat et al., 1998). One gene encoding basic fibroblast growth factor (bFGF) in *Xenopus* oocytes was located on the antisense strand of another bFGF gene (Kimelman & Kirschner, 1989). The complementary strand of heat shock protein HSP70 gene in *Achlya klebsiana* codes for an NAD-specific glutamate dehydrogenase (NAD-GDH) gene. The antisense strand of the coding region of HSP70 corresponded to the last and the largest exon of NAD-GDH gene and the antisense strand of the 3′untranslated region in HSP70 corresponded to the introns and exons of NAD-GDH gene (LeJohn et al., 1994).

Although the antisense strand of the 5′ untranslated region of cMeHRGP1 coded for part of PSAT, the antisense strand of the coding region of the HRGP was unlikely to code for any proteins due to its repetitive sequences and the abundance of serine whose antisense codon is a stop codon. What is possible though, is that the major part of the coding region of cMeHRGP1 may be an intron of the antisense gene. Genes existing in the introns of other genes or overlapping genes have been reported (Bonnelye & Laudet, 1994). In which case the 3′end of cMeHRGP1 may serve as the 5′untranslated region and the initial coding region of the antisense gene. A comparison of the deduced amino acid sequence of PSAT from the antisense strand with published PSAT sequences from other plants shows that this lacks about 50 amino acids from the amino terminus and about 180 from the carboxy terminus, the latter being beyond the cloned sequence. In other words, the antisense strand of cMeHRGP1 codes for the central 130 amino acids of PSAT. Therefore, an antisense message of the cMeHRGP1 clone could not code for a functional PSAT as it stands. Isolation of the cDNA and genomic clone(s) of PSAT from cassava would be needed to confirm that the antisense gene pairing with cMeHRGP1 encodes a functional PSAT.

The biological role of the transcript from the antisense strand or natural antisense RNA may lie in the regulation of the expression of the sense gene. A growing number of eukaryotic genes are thought to be regulated at least in part by natural antisense RNA transcribed from the presumptive non-coding DNA strand (Knee & Murphy, 1997). Modification of A to G was detected in the sense transcripts of fibroblast growth factor gene, which was thought to be the result of the action of modifying enzyme on the sense and antisense duplex. The modification may lead to an inactive polypeptide as the changes occurred in the region that was suggested to be crucial for binding to the bFGF receptor (Kimelman & Kirschner, 1989).

The deduced amino acid sequence of cMeHRGP1 showed typical repeat motifs found in the HRGPs of other plants. The SP_4 motif, which leads to molecular inflexibility and wall self-assembly, is the most common repeat motif in HRGPs. There are 28 SP_4 repeats in the cassava HRGP1. There are two SP_4-Y_3K-SP_4-SP motifs and eleven SP_4-Y_3H-SP_4-VK repeat units. SP_4-Y_3K motifs have been found in HRGPs of bean, tomato and potato, SP_4-Y_3H repeats in rape and soybean, SP_4-VK-SP_4in maize (Sommer-Knudsen et al., 1998), but the long and main repeat motif SP_4-Y_3H(or K)-SP_4-VK or SP_4-VK-SP_4-Y_3H(or K) has not been reported before to our knowledge.

The tyrosine (Y) in YXYK (X = any amino acid), Y_3H or Y_3K provides the possibility of both inter- and intra-molecular cross-linkages through the formation of isodityrosine (IDT), thereby increasing the rigidity and hydrophobicity of the molecules (Kieliszewski & Lamport, 1994; Sommer-Knudsen et al., 1998). This may strengthen the cell wall and form a barrier to prevent water loss or pathogen infection at wound sites. Palindromes PPPPSPPPP, YYY OR YIY were also present in the predicted polypeptide of cMeHRGP1. The same sequences were found in

other plants (Kieliszewski & Lamport, 1994). The palindromic peptides could create centrosystemetric domains, which act as self-assembly nucleation sites. In other words, an intermolecular interaction may establish a structure which can initiate a succession of intermolecular reactions or 'growth' (Kieliszewski & Lamport, 1994; Lindsey, 1991).

Increases in the level of HRGP transcripts upon wounding have been observed in many plants; for example, in potato tubers, carrot and tomato (Bown et al., 1993; Showalter et al., 1992; Tierney et al., 1988). Most of these HRGP genes have also been shown to be inducible upon pathogen infection; for example, wound-induced HRGP genes were induced by both compatible and incompatible pathogens in bean (Corbin et al., 1987). The special features of HRGP sequences and the increased expression of HRGP genes during wounding and pathogen infection imply a function in sealing off wound sites to prevent desiccation and/or penetration by pathogens. Proline-rich cell wall protein was rapidly insolubilised at wound sites mediated by H_2O_2 in soybean cells and bean cells incubated with a fungal elicitor (Bradley et al., 1992). Tomato HRGP insolublisation was detected in tomato cell suspension cultures immediately after challenging with a yeast elicitor, prior to the peak of reactive oxygen species, suggesting a primary defence mechanism incorporating pre-existing HRGPs, peroxidase and a peroxide generating system (Brownleader et al., 1997).

Cassava cultivar MDOM 5, though it is classified at CIAT as showing low susceptibility to post-harvest physiological deterioration (PPD), often exhibits a range of susceptibility; a frustrating feature common to many cassava varieties (M. Fregene, personal communication). MDOM 5 roots harvested from the University of Bath tropical glasshouse generally showed low susceptibility, yet the roots used for these experiments, which were harvested at CIAT, showed initial signs of PPD within 24 hours of harvest. In these roots mRNA corresponding to cMeHRGP1 did not accumulate until day three after harvest, by which time the visible symptoms of PPD were very marked. HRGPs and their insolublisation are a component of wound repair and are normally induced by wounding in other plants. Yet in the case of harvested cassava storage roots, the gene for cMeHRGP1 was induced late, well after the symptoms of PPD had reached an advanced state throughout the whole root. While HRGPs are only a minor component of a plant's total wound repair process, the fact that cMeHRGP1 mRNA accumulated late during the PPD reaction is additional support for the view that the deterioration process is closely linked to the inadequacy of the wound repair response in detached cassava storage roots. The mRNA detected was over 4000 bases, which was considerably larger than the 1696bp of cMeHRGP1. It is interesting to note that the parsley HRGP clone, ELI9, used to isolate cMeHRGP1 also detected a mRNA in parsley of about 4000 bases (Hirsinger et al., 1997). Suggesting that the cassava MeHRGP1 transcript is substantially larger than the cDNA presented here.

While cell suspension cultures may be an artificial system, they permit a higher degree of control over environmental variables than do field-grown roots; certainly suspension cultures have been widely used to dissect many aspects of a plant's response to biotic and abiotic stress, including the expression of HRGP genes (Hirsinger et al., 1997). The results presented here demonstrate that cassava cells from MCOL 22 (susceptible to PPD) are capable of a rapid induction of HRGP genes, with mRNA corresponding to cMeHRGP1 accumulating within 12 hours of being challenged with a glucan cell-wall elicitor (Figure 8). Therefore, the response of three days observed in the roots is very slow compared to the potential of cassava cells. Again, this is additional evidence that the delayed and inadequate wound healing observed during PPD is abnormal and does not represent the cassava plant's normal potential response.

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