Relevance of Class I α-Mannosidases to Cassava Postharvest Physiological Deterioration

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Supporting Information

ABSTRACT: Class I α-mannosidases (MNSs) play important roles in protein N-glycosylation. However, no data are currently available about MNSs in cassava (Manihot esculenta), of which the functions are therefore not known, particularly in relevance to postharvest physiological deterioration (PPD). A total of seven genes were identified from the cassava genome in the present study. Two (MeMNS2 and MeMNS6) of the seven genes may be pseudogenes, as indicated by sequence alignment and exon-intron organizations. Five MNSs could be classified into three subfamilies. Tissue-specific expression analysis revealed that MNS genes have distinct expression patterns in different tissues between sugar cassava and cultivated cassava varieties, indicating their functional diversity. A PPD response and defense model was proposed based on the transcription data of MNSs and genes involved in reactive oxygen species, signal transduction, and cell wall remodeling. The findings help in the understanding of PPD responses in cassava.

INTRODUCTION

N-glycosylation is a major posttranslational modification of proteins in all eukaryotic cells. It affects many important biological processes such as protein folding,4-7 enzyme activity,8,9 intracellular trafficking,5,7 and cell–cell communications.8

Class I α-mannosidases (MNSs; glycosyl hydrolase family 47) are involved in early N-glycan-processing reactions and in N-glycan-dependent quality control in the endoplasmic reticulum (ER).9 Structural Ca2+ is required for catalysis, and MNSs are sensitive to the inhibitor kifunensine.10 The mammalian and Arabidopsis class I α-mannosidase family consists of three protein subgroups: ER α 1,2-mannosidases I (ER-MNSIs), Golgi-α-mannosidases I (Golgi-MNSIs), and ER degradation-enhancing α-mannosidase (EDEM)-like proteins.9 These characteristics and classifications have been highly conserved throughout eukaryotic evolution. Five MNS proteins were characterized in Arabidopsis. MNS1, MNS2, and MNS3 played a role in root development and cell wall biosynthesis,8 while MNS4 and MNS5 were required for endoplasmic-reticulum-associated degradation of misfolded glycoproteins.11

Cassava is a major food crop in the tropics and subtropics as well as a potential biofuel crop due to its high starch production.12 Roots are the main storage organs in cassava. Cassava has a fibrous root system consisting of the adventitious roots that arise from the base of the stem cuttings and their attendant lateral roots that successively develop.13 The rapid development of both adventitious and lateral roots may last 8 or 10 weeks after planting and can reach a maximum weight in 60 days after planting.14 Only a few fibrous roots become tuberous and most of the others continue their function of nutrient absorption.15 The tuberous root is the organ that is harvested. However, rapid postharvest physiological deterioration (PPD), a unique phenomenon in harvesting storage roots, has become a major constraint for extending cassava shelf life in comparison with the roots of the other crops.16 PPD is a complex physiological and biochemical process, which involves many regulatory networks linked with specific proteins and signaling pathways.17 PPD, which occurs 24–48 h after harvest, is initiated by an oxidative burst,18 followed by the accumulation of secondary metabolites.19,20 Recent data suggested the crosstalk between reactive oxygen species (ROS), calcium signal transduction, and programmed cell death (PCD) during PPD in cassava.21 The most important limiting factors for studying PPD were the lack of PPD-tolerant varieties and the absence of accurate regulating factors known to alleviate cassava PPD.16 Functional studies are therefore needed to better understand the PPD response and provide a worthwhile approach to control it.

Due to the importance of MNSs in stress tolerance22 and root development of Arabidopsis, we were interested in
exploring its functions in relevance to cassava roots and PPD. It is reported that the suppression of the N-glycan-processing enzyme α-mannosidase can enhance the shelf life of fruits. Therefore, the identification and expression analysis of MNSs were conducted in the present study. The putative relationship between PPD and MeMNSs was also explored. The results will be helpful for further understanding of the possible roles of MNSs and build a foundation for future studies on cassava PPD.

■ RESULTS

Identification and Phylogenetic Analysis of MNSs in Cassava. The amino acid sequences of five Arabidopsis thaliana MNSs (AtMNS1 to AtMNS5) were used to search the cassava genome for candidate MNS proteins. Seven cassava (Manihot esculenta) enzymes with significant amino acid sequence similarity to AtMNSs were identified and named as MeMNS1-6, including MeMNS3-1 and MeMNS3-2. Five cassava MNS enzymes contain a conserved α-1,2-mannosidase sequence domain (PfamPF01532) and are members of glycosyl hydrolase 47 family (carbohydrate-active enzyme CAZy) database. However, MeMNS2 and MeMNS6 contained only a partial glycosyl hydrolase 47 domain.

To determine the evolutionary relationship among the MNS enzymes, sequences of 20 MNS family members from M. esculenta, A. thaliana, Brachypodium distachyon, and Oryza sativa were analyzed using neighbor-joining phylogenetic tree analysis. These MNS enzymes were classified into three subgroups (Figure 1). MeMNS1 was classified as Golgi-MNS.

Characterization of MeMNS Genes and Enzymes. Most of the full-length coding sequences of the MeMNS genes ranged from 1725 bp (MeMNS1) to 1890 bp (MeMNS3), whereas MeMNS2 and MeMNS6 were 969 bp and 687 bp, respectively. The size of most deduced MeMNS enzymes varied between 502 amino acids (aa) and 629 aa, except MeMNS2 (322 aa) and MeMNS6 (228 aa). The molecular weights (MW) of most MeMNS enzymes varied from 56.95 to 70.52 kDa, and the theoretical isoelectric points (pI) of the proteins encoded by these genes ranged from 5.21 to 7.00 (Table 1). All seven MeMNS sequences were aligned with AtMNS1, which showed that MeMNSs contain multiple conserved residues and relatively variable N-terminal and C-terminal domains (Supporting Information Figure S1). Interestingly, MeMNS2 contains the first half of the MeMNS1, while MeMNS6 contains the second half; consequently, both MeMNS2 and MeMNS6 would not be a functional MNSI.

The five MeMNSs were aligned with all AtMNSs (Supporting Information Figure S2). The structure of each MNS is shown in Figure 2 according to the subgroup. The similarity to AtMNS, except MeMNS2 and MeMNS6, suggested that all of the enzymes have four active site residues and one Ca2+ binding residue. Those active site residues are conserved. Taking MeMNS1 as an example, four active site residue numbers are 187, 319, 430, and 452, and one Ca2+ binding residue number is 536. However, MeMNS2 and MeMNS6 have only a partial GH47 domain. MeMNS2 has two active site residues, whereas MeMNS6 has two active site residues and one Ca2+ binding residue.

Structure Analysis and Chromosomal Distribution of MeMNS Genes. To gain further insight into the evolutionary relationships of MeMNSs, the exon–intron structure for each member was analyzed. The number of exons in MeMNS genes ranged from 5 to 17 (Figure 3A). MeMNS1 had 14 exons. Among the ER-MNSI group, MeMNS3-1 and MeMNS3-2 had six exons, and the exon–intron structures were similar. MeMNS4 and MeMNS5 had 17 and 15 exons, respectively. MeMNS2 had eight exons, while MeMNS6 had only five exons.

The chromosomal distribution and orientation of MeMNS genes were also identified. The results showed that the seven genes can be mapped to four chromosomes (Figure 3B). No tandem duplication of the cassava MeMNS genes was found. MeMNS1, MeMNS3-1, MeMNS3-2, and MeMNS5 had the same orientation and are present on chromosomes 3, 15, 17, and 6, respectively. MeMNS4 was found to be present on chromosome 3, while MeMNS2 and MeMNS6 were mapped to chromosome 6, with the opposite orientation as MeMNS1.

Expression Analysis of MeMNSs in Different Tissues and Root Developmental Stages. The cassava cultivars M. esculenta cv. SC no. 205 (SC205, with a high starch content approximately at 28%) and M. esculenta cv. CAS36.12 (sugar cassava, a low starch content approximately at 3%) were selected and assayed for the expression of MeMNSs in different tissues and at different root development stages. In SC205, MeMNS1, MeMNS3-1, and MeMNS3-2 were highly expressed in leaves, stem, and tuberous roots, respectively (Supporting Information Figure S3A). MeMNS4 and MeMNS5 were highly expressed in leaves. However, in CAS36.12, MeMNS1 was highly expressed in petioles, and the other four genes were highly expressed in tuberous roots (Supporting Information Figure S3B).
## Table 1. Basic Information of Seven MNS Family Genes in Cassava

| genes   | gene ID     | accession number | ORF length (bp) | protein length (aa) | theoretical pI | molecular weight (kDa) |
|---------|-------------|------------------|-----------------|---------------------|----------------|------------------------|
| MeMNS1  | Manes.03G080300 | MK000551         | 1725            | 574                 | 5.92           | 65.07                  |
| MeMNS2  | Manes.06G018500 | OAY46678         | 969             | 322                 | 5.44           | 36.22                  |
| MeMNS3-1| Manes.15G120800 | MK000552         | 1890            | 629                 | 6.17           | 70.52                  |
| MeMNS3-2| Manes.17G099200 | MK000553         | 1890            | 629                 | 7.00           | 70.34                  |
| MeMNS4  | Manes.03G169900 | MK000554         | 1866            | 502                 | 5.21           | 56.95                  |
| MeMNS5  | Manes.06G101300 | MK000555         | 1731            | 576                 | 5.76           | 65.84                  |
| MeMNS6  | Manes.06G018400 | OAY46677         | 687             | 228                 | 5.56           | 26.28                  |

Figure 2. Schematic representation of AtMNSs and MeMNSs. ■ indicates the transmembrane domain, silver box solid shows the GH47 domain. The number means four active site residues (from left) and Ca^{2+} binding residue (the last).

Figure 3. Structure analysis and chromosomal distribution of the MeMNS genes. (A) Exon–intron structure of the seven MeMNS genes. Introns and exons are shown in blue lines and green boxes, respectively. (B) Chromosome localization of MeMNS genes. The position of the MeMNS genes is shown in red lines with the orientation indicated in blue arrows.
The expression level of MeMNSs was measured during root development 4, 6, 8, and 10 months after planting (Supporting Information Figure S3C,D). In SC205, the expression level of MeMNS1 was similar in months 4 and 6 but dropped by 90% in months 8 and 10. On the contrary, in Cas36.12, the level of MeMNS1 expression dropped only by 50% in months 6, 8, and 10, relative to month 4. In SC205, MeMNS3-1 and MeMNS3-2 expressions were considerably lower in month 8 and increased again to 50 and 100% of month 4 for MeMNS3-1 and MeMNS3-2, respectively. In CAS36.12, MeMNS3-1 and MeMNS3-2 expressions were low in months 6 and 8 and the same in months 4 and 10, respectively. Interestingly, the EDEM1 MeMNS4 and MeMNS5 showed the same expression pattern within each cultivar.

**N-Glycoprotein Detection and Transcriptome Analysis of MeMNSs and Related Genes After Harvest.** The cassava cultivars *M. esculenta* cv. SC no. 14 (SC14) (with high PPD resistance) and *M. esculenta* cv. SC no. 9 (SC9) (PPD sensitivity) were selected and examined for differences in glycoprotein expression and transcriptome analysis. The original images of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot are shown in Figure 4A. The results showed that the number of N-glycoproteins in SC9 tuberous roots at 48 h was fewer than that in SC14.

To better understand the expression patterns of MeMNS genes in response to PPD, RNAs from tuberous roots in different times were extracted for subsequent RNA-seq analysis. Transcripts of five MeMNSs and other related genes were captured in a transcriptional data search. The differential expressions of these genes in SC14 and SC9 at 48 h were compared to those at 0 h. The analyses revealed some upregulation of genes that are associated with ROS, signal transduction, and cell wall remodeling in SC9 and SC14 tuberous roots (Figure 4B). It showed upregulation of genes at 48 h that encode antioxidation enzymes in SC14 tuberous roots, such as superoxide dismutase (SOD), peroxidase (POD), and glutathione peroxidase (GPX). Moreover, the expressions at 0 and 48 h in SC9 varied notably larger than those in SC14. Calmodulin (CaM) and 14-3-3 showed a similar pattern as SOD. The expression of ethylene biosynthesis gene encoding S-adenosylmethionine synthase (SAMS) was downregulated at 48 h, whereas the jasmonic acid biosynthesis gene encoding alleneoxidasecyclase (AOC) was upregulated.

The RNA-seq data also showed changes in genes that encode certain cell-wall-degrading enzymes, such as pectin methylesterase (PME) and glucan endo-1,3-β-d-glucosidase (TomQb). Therefore, PPD not only caused ROS formation but also affected pectin and cellulose degradation. Moreover, most MeMNSs were downregulated 48 h after harvest, whereas MeMNS3-1 expression was upregulated in SC14 but downregulated in SC9. MeMNS3-1 is suspected to be the main factor gene in PPD resistance process. A PPD response and defense model presented in Figure 5 illustrates the regulation of ROS, signal transduction, programmed cell death (PCD), N-glycosylation, and cell wall remodeling in cassava PPD processes.

**DISCUSSION**

**MeMNS2 and MeMNS6 May Be Pseudogenes.** The ORF lengths of MeMNS2 and MeMNS6 are 969 bp and 687 bp, respectively, and the corresponding enzymes have 322 aa and 228 aa. An apparent insertion of some nucleotides makes it two different genes, which is supported by the structure distribution (Figure 2) and the location in chromosome 6 (Figure 3B). Interestingly, MeMNS2 and MeMNS6 reside very close in the chromosome. If MeMNS2 and MeMNS6 were linked together, it would closely resemble MeMNS1. Although both of them had a partial sequence of GH47 family and expressed in the transcript level, it may not produce a functional, full-length protein. According to the definition of pseudogenes,24 MeMNS2 and MeMNS6 may be pseudogenes in this family, while the final confirmation of this assignment has to await the demonstration of the appropriate enzymatic activity.

**Potential Role of MeMNSs in PPD.** The PPD phenomenon is a global challenge for cassava breeders, farmers, and factories. It can cause cassava root spoilage and reduce the shelf life during storage.25 Wounding during harvest rapidly leads to increased ROS in cassava tuberous roots. Insufficient endogenous ROS scavenging thus results in excess ROS, which triggers rapid PPD responses.26 Therefore, increasing the production of ROS scavengers is a key regulator of PPD. The expression of SOD and CAT,26 mitochondrial alternative oxidase (AOX),15 glutathione reductases,17 and GPX18 during PPD lead to timely scavenging of excess ROS,
thereby maintaining the ROS homeostasis balanced and delaying PPD occurrence (Figure 5).

Calcium (Ca\(^{2+}\)) plays an important role in signal transduction and activates a collection of target proteins leading to a physiological response. Ca\(^{2+}\) is also necessary for the integrity of the cell wall and the membrane systems.\(^{29}\) In previous studies, Ca\(^{2+}\) was linked with regulating PPD onset. Following root wounding, calcium changes preceded a burst in ROS.\(^{30}\) In the present study, MeMNS genes were downregulated at 48 h (Figure 4). This may cause more Ca\(^{2+}\) signatures being recognized by several types of Ca\(^{2+}\)-sensor proteins, such as calmodulins (CaMs)\(^{31,32}\) (Figure 5). The Ca\(^{2+}\)−CaM complex may activate target proteins to resist PPD. Further elucidation of roles of MNSs binding with Ca\(^{2+}\) specifically with PPD would advance the understanding of the molecular machinery controlling PPD onset.

The cell wall undergoes significant remodeling during PPD.\(^{33}\) PME contributes to cell wall loosening and degradation by promoting the activity of cell wall hydrolases.\(^{33}\) In the present study, PME increased in SC14, the PPD-resistant variety, from 0 to 48 h (Figure 4B), whereas PME in SC9 decreased. N-glycoproteins are a constituent of the cell wall.\(^{34}\) The data showed that suppressing N-glycan-processing enzymes can enhance the fruit shelf life, such as α-mannosidase and β-D-N-acetylhexosaminidase, which are involved in degrading cell wall N-glycoproteins.\(^{35}\) Transcriptomic analysis showed that PPD affects MeMNS genes that are responsible for protein N-glycosylation. The ER and Golgi-MNSs genes were expressed higher in the PPD-tolerant variety SC14 than those in the PPD-sensitive variety SC9. It appears that the modification of N-glycan-processing ability would be a potential strategy to extend the cassava root shelf life.

On the basis of the data described above, we would propose a mechanism of PPD response and defense in cassava (Figure 5). All impact factors associated with PPD were used to construct the pathways. The gray rectangles represent proteins.

![Figure 5. Proposed regulatory pathways of PPD response and defense in cassava tuberous roots. The MNSs identified in this study and other proteins related to PPD were used to construct the pathways.](image)

**CONCLUSIONS**

A total of seven class I α-mannosidase genes (MNSs) were identified from the cassava genome. Two (MeMNS2 and MeMNS6) of the seven genes may be pseudogenes. Five MNS enzymes could be classified into the three enzyme subfamilies Golgi-MNSI, ER-MNSIs, and EDEMs. MeMNS1 was classified as Golgi-MNSI; MeMNS3-1 and MeMNS3-2 as ER-MNSIs; and MeMNS4 and MeMNS5 were classified as EDEMs. However, MeMNS2 and MeMNS6 were far away from AtMNS1 and AtMNS2. The MNS genes showed distinct expression profiles in different tissues. A PPD response and defense model was proposed on the basis of the transcription data of MNSs and genes involved in ROS formation, signal transduction, and cell wall remodeling. Class I α-mannosidases are putatively relevant to cassava postharvest physiological deterioration.

**MATERIALS AND METHODS**

**Plant Materials.** Cassava cultivars with high starch content (~28%) (M. esculenta cv. SC no. 205 (SC205)), low starch content germplasm (~3%) (sugar cassava, M. esculenta cv. CAS36.12), high PPD resistance variety (M. esculenta cv. SC no. 14 (SC14)), and PPD-sensitive variety (M. esculenta cv. SC no. 9 (SC9)) were selected in the present study. SC14, SC9, and SC205 were obtained from the Tropical Crops Genetic Resources Institute (TCGRI) and the Chinese Academy of...
Tropical Agricultural Sciences (CATAS), and CAS36.12 originated from Brazil (Embrapa) and was currently being planted in the Cassava Germplasm Bank (CGB), TCGRI, CATAS. For the analysis of gene differential expression in tissues and organs, the plant materials from SC205 and CAS36.12 were collected as follows. The leaves (L), petioles (P), stems (S), tuberous roots (T), and fibrous roots (F) were collected 60 days after planting. For differential expression analysis of these genes during tuber root development, the roots were collected at 120 (4 months), 180 (6 months), 240 (8 months), and 300 days (10 months) after planting. Biological samples were collected in triplicate for analyses. All materials were immediately frozen in liquid nitrogen and stored at −80 °C for RNA isolation.

Identification of MNS Gene Family in Cassava. The sequences of five A. thaliana MNS proteins were downloaded from the TAIR10 database (http://www.arabidopsis.org). BLASTP searches were performed in the cassava genome database (http://www.phytozome.net/cassava) with the Arabidopsis MNS proteins as queries. First, all corresponding protein sequences of the putative MeMNS family members that satisfied $E < 10^{-10}$ were downloaded. All candidate proteins were then tested with the Pfam database (http://www.phytozome.net/cassava) with the protein information on Uniprot. Multiple sequence alignments of MeMNS genes were tested with the Pfam database (http://www.phytozome.net/cassava) with the protein information on Uniprot.

Multiple Sequence Alignment and Phylogenetic Analysis of MNS Proteins. Multiple sequence alignments were performed using ClustalW with gap open and gap extension penalties of 10 and 0.1, respectively. Amino acid sequences of 20 proteins from M. esculenta, A. thaliana, B. distachyon, and O. sativa were aligned using ClustalX2.0, and the unrooted phylogenetic tree was generated using MEGA 4.0 software, with the neighbor-joining method and bootstrap values from 1000 replicates at each branch. The full-length cDNA sequences of MeMNS genes were downloaded from the cassava-sequencing database.

Protein Properties and Sequence Analyses. The MW and pI of presumed MeMNS proteins were predicted with the Prot Param online tool. The protein structures were drawn using the protein information on Uniprot.

Exon–Intron Structure Analysis and Chromosomal Mapping. The exon–intron structure of each MeMNS gene was determined by aligning the cDNA sequence of MNS with the genomic sequence in the cassava genome database. The gene schematic structure was drawn by the gene structure display server. The genomic position of the MeMNS genes and the total length of each chromosome were obtained from the cassava genome database. Subsequently, the MeMNS genes were manually mapped onto chromosomes.

Cloning of Five MeMNS Genes. MeMNS1, MeMNS3–1, MeMNS3–2, MeMNS4, and MeMNS5 were cloned in the present study. The total RNA was extracted from cassava leaves using RNA Plant plus Reagent (TianGen, Beijing, China). Full-length cDNAs of the MeMNS genes were isolated by reverse transcription PCR using gene-specific primers (Supporting Information Table S1). All PCR products were separately cloned into the pEASY Blunt vector (TransGen) and were sequenced by Sangon Biological Engineering Technology and Services (Shanghai, China).

Quantitative Real-Time PCR (qRT-PCR) Analyses. RNA samples were extracted with an RNAprep Pure Plant plus Kit according to the manufacturer’s protocol (Tiangen). The RNA quality was determined by running an agarose gel with GelStain (TransGen) staining. The RNA concentration was determined with NanoVue Plus ultramicro spectrophotometer (GE Healthcare). Reverse transcription was performed according to the manufacturer’s protocol (TransGen). The primers are listed in Supporting Information Table S2. The qRT-PCR reactions were performed in 10 μL volume in a Piko REAL thermocycler (Thermo Fisher Scientific Inc, Göteborg, Sweden). The formula $2^{-ΔΔCt}$ was used to calculate the relative fold change between the treatment stages. The experiments were performed in triplicate.

Western Blot Analyses. The roots of the cassava genotypes SC14 and SC9 were carefully harvested 10 months after planting. The halved roots in uniform size were randomly stored at 26–28 °C and 70–80% relative humidity. After 0 and 48 h, the total proteins were extracted from injured tuberous roots according to An et al. Proteins were detected by immunostaining with anti-HRP antibody (Gtxot-096-D) from ImmunoReagents, Inc. (Raleigh, NC). Western blots were developed according to the method of NBT/BCIP from Roche (11681451001). The signals on the nitrocellulose were scanned immediately by Image Scanner III (GE healthcare), and the data were quantified with ChemiImager 4400 software.

Transcriptome Analysis. The total RNA from tuberous roots was extracted at 0 and 48 h. RNA-seq technique was employed to measure the global expression of cassava genes. Clean reads were mapped to the cassava reference genome (version 6.1), derived from the Phytozome12 website, using TopHat v.2.0.10 (http://tophat.cbcb.umd.edu/). The resulting alignment files were provided as input for Cufflinks to generate transcriptome assemblies. DESeq was applied to identify differentially expressed genes with a random sampling model based on the read counts for each gene.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b03558.

Multiple sequence alignment of protein sequences corresponding to the class I mannosidases and sequence relationship to other processing mannosidases (Figure S1); multiple sequences alignments of five MeMNSs identified in cassava with the Arabidopsis MNS proteins as queries (Figure S2); expression analysis of MeMNS genes in different tissues and different root developmental stages (Figure S3); gene-specific primers of five MeMNSs used for RT-PCR amplification (Table S1); and gene-specific primers of MeMNSs used for qRT-PCR amplification (Table S2) (PDF)

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**Notes**

The authors declare no competing financial interest.
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