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Cloning and Molecular Characterization of α- and β-Amylase Genes from Cassava (*Manihot esculenta* Crantz)

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ABSTRACT

Starch is the primary storage polysaccharide in plants and can be degraded by amylolytic enzymes. Starch-degrading enzymes can be classified into α-amylase (1, 4-α-D-glucan glucanohydrolase; EC 3.2.1.1) and β-amylase (1, 4-α-D-glucan glucanohydrolase; EC 3.2.1.2). Two clones encoding α-amylase and β-amylase from leaves of Thai cassava were isolated and sequenced. The partial clones were 705 and 1,960 nucleotides in length, respectively. Total RNA was extracted from several parts of the cassava plants and analyzed for the presence and level of expression of the two genes by using PCR DIG-labeled α-amylase and β-amylase probes. The highest expression of the α-amylase gene was observed in storage roots, lower but detectable expression was in leaves and stems. On the other hand, the β-amylase gene was observed for highly expressed in leaves and storage roots. The copy number of these genes was determined using Southern analysis indicated that α-amylase is a single copy gene, while β-amylase is present in multiple copies in the cassava genome.

Key words: cassava, starch degradation, α-amylase and β-amylase

INTRODUCTION

Several pathways of starch degradation exist and amylases are thought to play an important role. In various plant species the role of amylases has been reported but not in the tropical root crop cassava. Starch can be cleaved by a series of hydrolytic enzymes known as amylases. α-amylase (1, 4-α-D-glucan glucanohydrolase, EC 3.2.1.1) catalyzes the internal cleavage of glucosyl bonds, giving rise to small-molecular-mass glucans called limit dextrins as well as glucose and maltose. β-amylase (1,4-α-D-glucan maltohydrolase, EC 3.2.1.2) cleaves starch from the non-reducing end resulting in maltose as the end product. Maltose, short-chain glucans and maltosaccharides or limit dextrin can be further degraded to glucose molecules by the action of α-glucosidase. These three enzymes, combined with debraching enzyme, can effectively degrade starch to glucose (Buchanan *et al*., 2000).

α-amylase has been shown to be localized in the aleurone tissue (Yu *et al*., 1991), chloroplasts and cytoplasm. β-amylase is localized in the stroma of mesophyll cell chloroplasts (Lao *et al*., 1999; Scheidig *et al*., 2002), the vacuole and the cytoplasm. Both types of amylases occur widely in higher plants and also some microorganisms (Hyun

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and Zeikus, 1985). Similar to α-amylase, β-amylase can bind and attacks starch granules, but generally, α- and β-amylases do not effectively hydrolyze raw starch granules, because starch granules are resistant to amylolytic digestion (Hyun and Zeikus, 1985). Therefore, starch degradation with amylases essentially requires a long period of time (24 to 72 hours) at an elevated temperature.

Expression role of α-amylase genes is regulated at the transcriptional level both positively (by gibberellic acid) and negatively (by abscisic acid; Buchanan *et al*., 2000). Individual rice α-amylase genes have been shown to be differentially expressed in the germinating seed and in various tissues of the rice plant. In addition, the level of rice α-amylase gene expression was markedly different between callus and the germinating seed. The expression of α-amylase genes in rice suspension culture cells derived from immature embryos is greatly induced by the deprivation of carbohydrate nutrient (Lue and Lee, 1994). β-amylase activity is abundant in both photosynthetic and storage organs of many plant species (Scheidig *et al*., 2002). The primary function of β-amylase is involvement in starch breakdown in plants (Kossmann and Lloyd, 2000). The mode of regulation of plant β-amylase genes is complex and debate is ongoing about its specific role. Accumulation of β-amylase mRNA is regulated by sugars (Gana *et al*., 1998). It was found that β-amylase mRNA of *Arabidopsis* increased in rosette leaves when the whole plant or excised, fully expanded leaves were supplied with sucrose, glucose or fructose. However no effect was found for mannitol or sorbitol (Mita *et al*., 1995). Likewise, expression of β-amylase in sweet potato could occur in darkness if leaf-petiole cuttings were supplied with sucrose (Nakamura *et al*., 1991). In addition, dipping sweet potato leaf-petiole cuttings in poly-galacturonic acid or chitosan also induced β-amylase mRNA accumulation, whereas mechanical wounding of leaves only occasionally induced β-amylase gene expression (Ohto *et al*., 1992). In general β-amylase expression and activity are regulated by light (Tepperman *et al*., 2001), sugars (Mita *et al*., 1995), phytohormones (Wang *et al*., 1996), proteolytic cleavage (Sopanen and Lauriere, 1989), and abiotic stresses; such as salt, cold and heat (Seki *et al*., 2001).

Elucidation of the regulation of starch degradation of cassava tubers has become our main research focus. To this end we are isolating and characterizing the genes encoding the enzymes responsible for these processes. The integration of basic knowledge in cassava physiology, biochemistry and molecular biology will develop a new body of knowledge to understand the regulation of starch degradation and will certainly assist the breeding program to develop the traits of interest in a shorter timeframe and at lower costs.

Thus, understanding the changes in the processes related to starch breakdown during tuberous root development, especially in the last period before harvest would enable us to identify the major factors affecting the quantity and quality of starch.

MATERIALS AND METHODS

Plant materials

Three varieties of cassava R1, KU50 and HB60 were used. R1 was developed from local varieties. It is well adapted and can be planted in all environmental conditions, including low- nutrient soil and prolonged dry periods. However, when compared with the other cultivars used in this study it has a low starch percentage (19.2%). KU50 is the most popular variety (cultivated in about 56% of the total cassava planting area). It contains a high starch percentage (23.7%). Huayi Bong 60 (HB60), recently released by Kasetsart University, has an even higher starch percentage (25.4%) (Vichan *et al*., 2003) than KU50 and R1 (Sarakarn *et al.*, 2002 and Rojanaridpiched *et al.*, 2002). The plant- cuttings were grown under the

field conditions as outdoor experiment in the 0.1 m^3-pot containers at Agronomy Field Crop, Kamphaeng Saen Campus, Kasetsart University.

Genomic DNA extraction and total RNA extraction

Young cassava leaves were used for genomic DNA extraction essentially as described by Dellaporta *et al*. (1990). Total RNA was extracted from cassava leaves, storage roots and stems using the method from Salzman *et al*. (1999), Since the cassava samples of 6-week-old plants were used. And this research experiment in the Plant Physio-molecular Biology Laboratory, Center for Agricultural Biotechnology.

Polymerase chain reaction (PCR)

Polymerase chain reaction was used to amplify the α-amylase and β-amylase genes using degenerated primers. Primer sequences used for the isolation of the cassava sequence are given (Table 1). Primers were designed using highly conserved regions in α-amylase from *Arabidopsis*, *Vigna* and potato; and for β-amylase from *Arabidopsis*, soybean and sweet potato. The reaction was performed in the DNA thermal cycler (model 2400; PerkinElmer.) without mineral oil. After an initial denaturation at 94°C for 5 min, the PCR profile was set as follows: 1 minute of template denaturation at 94°C, 90 of primer annealing at 50°C for β-amylase gene, at 49°C for α-amylase gene and 2 min of primer extension at 72°C for a total of 35 cycles with a final extension at 72°C for 10 min. The PCR products were loaded on a 1% agarose gel with ethidium bromide to determine the sizes of the amplified products.

Cloning and sequence analysis

The PCR product of expected size was cloned into the pGEM-T easy vector according to the protocol described by the manufacturer (Promega). Plasmid pGEM-T easy was sequenced to confirm the direction and the correction of the insert using ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystem). The sequence was determined at Macro Gen Company, Seoul, Korea. GenBank nucleotides databases were searched for sequences having homology with α-amylase and β-amylase sequences using BLASTN program (NCBI). Comparisons of all nucleotide sequences were done using the MegAlign program (Lasergene).

Genomic Southern analysis

Genomic DNA was extracted from cassava leaves. The DNA (20μg) was digested separately

Table 1 Primers used in the isolation of a- and b-amylases coding sequence from cassava.

Primer	Sequence
α-amylase	
A1/1F	5'-GCGGACATAGTCATTAACCA-3'
A2/1R	5'-CCCTGGGTGTGTAAGAATG-3'
β-amylase	
B2F	5'-GCYMTYGAGATRTACAGKGA-3'
A1bF	5'-GGGGATGAGATCCTTGATGA-3'
A11bF	5'-GGTGAGAATGCACTTCCAAG-3'
B2R	5'-GCRATMGGMCGRTAMCCRTCT-3'
A2bR	5'-CTTGGMAGTGCATTYTCWCC-3'
A22bR	5'-TCCTCCTTCACCTTCAGCAC-3'

Remark: Y=C/T, M=A/C, R=A/G, K=G/T and W=A/T

with *Bam*HI, S*al*I and X*ho*I. Fragments were separated on a 1% agarose gel and transferred overnight to nylon membrane using 10X SSC according to the manufacturer's (Boehringer Mannheim) instructions. The DNA was cross-linked to the membranes by baking at 120°C for 30 min. The membranes were pre-hybridized and hybridized as described by the manufacturer (Boehringer Mannheim) using α-amylase and β-amylase probes. The signal was detected by chemiluminescence using CDP-star (Roche).

Dot blot hybridization

The six-week old intact cassava plants were treated with 5% sucrose by submerging the roots in the sugar solution. The plants were cultured for 2 days under continuously light/dark (10/14 hrs) periods. Mature leaves, stems and storage roots were harvested for two consecutive days. The tissues were cut into small pieces, frozen in liquid nitrogen and kept at -80°C until use.

Ten microgram of total RNA from different parts of cassava tissues was used for dot on Nylon membranes (Roche) which were baked at 80°C for 30 min. The membranes were pre-hybridized in high concentration hybridization buffer (7% SDS, 50% deionized formamide, 5X SSC, 2% blocking reagent, 50 mM sodium phosphate, pH 7.0, 0.1% N-lauroylsarcosine) at 50°C for 1 h, followed by hybridization with α-amylase and β-amylase probes. The hybridization was carried out overnight at 50°C in high SDS concentration hybridization buffer (similar to pre-hybridization buffer). The membranes were washed twice with 2X washing solution (2X SSC, 0.1% SDS, pH 7.0) at RT for 15 min and with 0.5X washing solution at 68°C for 15 min, respectively, before the detection.

RESULTS

Cloning, sequencing and characterization of α-amylase and β-amylase genes

α-amylase and β-amylase genes were cloned from cassava leaves using primers designed to compass highly conserved regions of α-amylase and β-amylase (Table1), respectively. Sequencing revealed that the partial α-amylase and β-amylase clones were 705 and 1,960 nucleotides in length, respectively (Figures 1 and 2). The complete nucleotide sequences of α-amylase and β-amylase were 3,473 and 4,772 bp length based on *V. mungo* and sweet potato, respectively. The partial nucleotide sequences of α-amylase and β-amylase gene were deposited in the GenBank database with accession number AY944583 and AY944584, respectively. The sequences were blasted and aligned by the program BLASTX and CLUSTALW .

Differential expression of α-amylase and β-amylase genes in cassava

Three different tissues of the Thai cassava

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281   TTTATTCGGA AAGTTTTCAT TTACTGAAAG CGGTGGATAA GGTTGGTTTT CATTCGTGTT AAGTTTGCCA
351   TAAGTTAAGT TTCAAAGTAT GGAAAGGAAT AAATCGAGAA AGTGTCAAAA GTTACAATCA GAGAATACTG
421   GGACACTCGA ACTACAAGGG CCAACTTGGA TTACAAACAA CATGTCACAG GTAAGGAAAG TTGACAGCGC
491   GGGAAAACTT TCAAATGAAT TCGATGTTAC AACAAAAGCA AACATTCAAG AATCAGTCAG AAGAGAATTT
561   GGCGTTTGCG TCGCAGGGCA GCCTTTAGCC TTGTTCAAAG ATGGTGACAT TCTAGGGCAG TCACTTCTTT
631   TGATTTAGGA CACTGGATCA ACTCGAGGCA CACTTGCCTC TTCTGCACGT CACATTATGA AGGGATATGA
701   ATACT
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Figure 1 Nucleotide sequence of a partial cassava α-amylase clone. Numbers on the left refer to nucleotide residues.

variety HB60, KU50 and R1 including leaves, stems and storage roots were used for total RNA extraction. Dot blot hybridization was used to determine the differential expression of α-amylase and β-amylase in each plant part (Figure 3).

Figure 3A shows the level of mRNA for α-amylase in leaves, stems and storage roots in response to treatment of plants with 5% sucrose.

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1       GCCCTCGAGA TGTACAGTGA CTACATGAGG AGCTTCAGGG AGAACATGTC AGATTTGTTA GAAGCTGGCC
71      CACAAACTCA AGGATGGGTT TTTCCTGGCA TTGGAGAATT CATTGTAAGC TTCAGAAATT ACATCACTAT
141     AAATTTTGAT TGGCATCTTA GCTTCTCCTT CTCCAATCAA CATTGCCTAA CATTTATCTC TAGTGCTATG
211     ATAAATATCT CAAAGCAGAA TTCAAAGAGG CAGCAGCAAG GGCAGGCAAC CCTGAATGGG AATTGCCTGA
281     TGATGCAGGG GAATACAATG ACACACCAGA AAACACTGGC TTCTTTAAAC CAAATGGAAC ATACCGTACT
351     GAAAAAGGAA ACTTCTTCTT GATATGGTAT TCCAACAGAT TATTGATCCA TGGGGATGAG ATCCTTGATG
421     AACCCATAAA GCATTCTTGG GCTGTAAAGT CAAAATAGCA GCTAAAGTAT ATCTCAAAAT GTTATTACTT
491     TCTTCGCTAA TCAGTAAAAA CGAAAAGCAT ATGTTCTTAT CCCAGTCTAT TATCAAACAG GTATCTGGAA
561     TTCATTGGTG GTACAAAGTT GATAATCATG CTGCAGAGCT TACAGCAGGA TATTACAACT TGCATGAAAG
631     AGATGGTTAC CGTCCTATTG CCAGGATGCT ATCAAGGCAT CATGCTATTT TGAATTTCAC ATGCCTTGAG
701     ATGAAGGACG CAGAGCAACC TGAAAATGCC AAAAGTGGAT CTCAGGAACT TGTTATGCAG GTAAAAAAGC
771     TTTAATTTAC ATGCTTTTCT TCCCTGATAA ATTTCAAACA CTTAGCGTGA TGAACTTAAG TTCTCAAAAT
841     GGAATTACAC TTGCACACTA ATGCAGCAAA TCTAAGAAAG GTAAAACCTA ACTATTATGC AAGCAGATAG
911     AGATAAATCA CACATATTTT CAAATTAATT CTACTCATTG TAAGTGCAGG TTTTGAGTTG GAGCTTGGAG
981     AGAGAATATT GAGGGTTGCA AAATGTTATT ACTTTCTTCG CTAATCCAGT CAAACCGATA AGCATATGAT
1051    TCTTATCCCA GTCTATTATC AAAACAGGTA TCTGGAATTC AATTGGTGGT ACAAAGTTGA TAATCATGCT
1121    GCAGAGCTTA CAGCAGGATA TTACAACTTG CATGAAAGAG ATGGGTACCG ACCCATTGCC CAGGATGCTA
1191    TCAAGGCATC ATGCTATTTT GAATTTCACA TGCCTTGAGA TGAAGGACGC AGAGCAACCT GAAAATGCCA
1261    AAAGTGGATC TCAGGAACTT GTTATGCAGG TAAAAAAGCT TTAATTTACA TGCTTTTCTT CCCTGATAAA
1331    TTTCAAACAC TTAGCGTGAT GAACTTAAGT TCTCAAAATG GAATTACACT TGCACACTAA TGCAGCAAAT
1401    CTAAGAAAGG TAAAACCTAA CTATTATGCA AGCAGATAGA GATAAATTCA CACATATTTT CAAATTAATT
1471    CTACTCATGT AAGTGCAGGT TTTGAGTGGA GCTTGGAGAG AGAATATTGA GGTTGCAGGT GAGAATGCAC
1541    TTCCAAGATA TGATGCGACA GCTTACAATA AAATCCTTTT AAATGTCAGG CCTAATGGTG TCAACAAGAA
1611    TGGCCAACCA AAACTGAGGA TGTATGGGAT GACATACCTA CGGTTATCTG ATGATCTATT AGAAGAAACC
1681    AATTTTAAGC TATTCAAGAC ATTTGTGAGG AAAATGCATG CAGACCAGGT AACCTGGCTT TTGTACAAGG
1751    ACATAAAATG ATCTGTATGT TCATTTTCAT TCACCTATCT GACATTAATT TTTATTTTCA GGATTATTGT
1821    CCAGATCTCA AGAAATACAA CCATGAAATA CGTCCACTGG AGCGGTCAAA GCAACAGATT CCAACTGAAG
1891    TTCTAACAGA AGCAACTAAG GCATTGGCAC CCTTCCCATG GGATAAAGAG ACAGACATGC CGGTGGATGG
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Figure 2 Nucleotide sequence of a partial cassava β-amylase clone. Numbers on the left refer to nucleotide residues.

A significant increase in the level of mRNA for α-amylase could be observed after treating the plant with 5% sucrose for 1 and 2 days in both leaves and storage roots. Clearly, the α-amylase in storage roots could be induced with sucrose which is reflected by the increased level of transcript accumulation after 2 days of treatment. The expression of α-amylase in stems of all three varieties demonstrated similar levels except for variety R1 at day 1 which showed a positive result. Similar to the accumulation level of α-amylase, the expression of storage root β-amylase increased with the time course of 5% sucrose treatment (Figure 3B). However, the β-amylase transcripts of leaves demonstrated a decrease trend in HB60 and KU50. The expression of β-amylase was undetected in the stems of every treatment.

Determination of α-amylase and β-amylase genes copy number

Genomic DNA was extracted from young leaf tissue of Thai cassava. Southern analysis was used to determine α- and β-amylase genes in cassava genome. The isolated genomic DNA migrated as a single band with a size of approximately 10 kb. *Bam*HI, S*al*I and X*ho*I endonucleases were used alone to completely digest cassava genomic DNA. Using the α-amylase probe, a single band was observed in S*al*I and *Bam*HI digested samples (Figure 4A). When the blot was probed with the β-amylase probe, the X*ho* I treated sample resulted in two bands. The B*am*HI sample gave three bands whereas the S*al* I sample showed a single band (Figure 4B).

DISCUSSION

Cassava starch which is accumulated in storage roots can also be degraded by the influence of hydrolytic enzymes. In this study, two amylase genes were characterized and molecularly isolated from cassava. Partial sequences of DNA corresponding to α-amylase and β-amylase genes were obtained by PCR of genomic DNA isolated from leaves. The β-amylases in the vegetative tissues of plants is most probably located outside

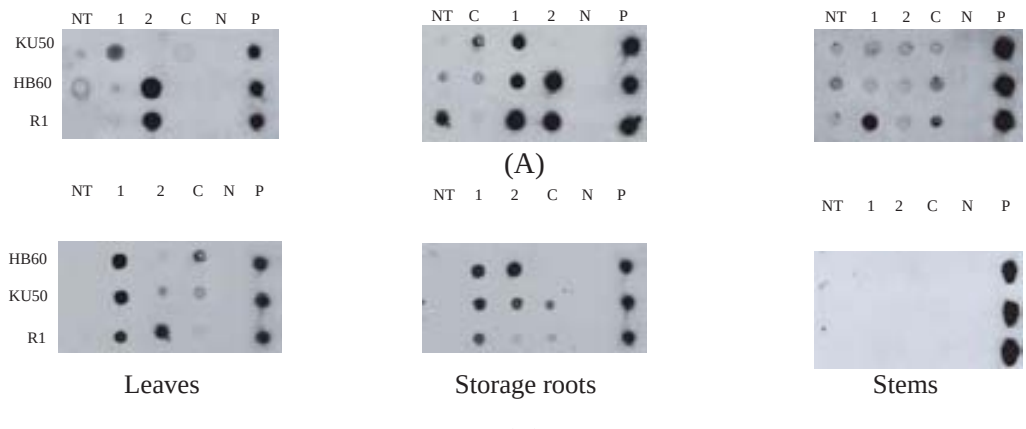

Figure 3 Induction of α-amylase (A) and β-amylase (B) genes expression in leaves, storage roots and stems of different Thai cassava cultivars using 5% sucrose. Roots of 6-week-old plants were dipped in a solution of 5% sucrose in water and incubated for 1 or 2 days before harvest. Dot blot hybridization was carried out using 10 μg of total RNA samples electrophoreses and probed with α-amylase; NT = non-treated (freshy), 1 = treated sucrose 1 day, 2 = treated sucrose 2 day, C = control, N = negative control and P = positive control, respectively.

the chloroplasts where the synthesis and accumulation of starch occurs (Beck and Ziegler, 1989) but the physiological roles of such β-amylases are not clearly understood. Caspar *et al*. (1989) reported that the level of β-amylase in rosette leaves of *Arabidopsis* was very high in certain mutants with altered starch metabolism under conditions associated with high-level accumulation of soluble sugars in leaves.

The sugar-inducible expression of the gene for α-amylase and β-amylase occurred not only in leaves, but also in stems and storage roots. The results indicated that expression of the multi-copied genes for β-amylase and single copy for α-amylase in cassava (Figure 3) are inducible in various vegetative tissues by a carbohydrate-mediated metabolic signal. They also suggested that the expression of these genes in various tissues may be regulated by the carbon partitioning and sink-source interactions in the whole plant (Mita *et al*., 1995).

In this study, we observed the differential expression of amylase genes in several parts of cassava. Among three parts of the plant; leaves, stems and storage roots, β-amylase expression was highest in the leaves. Whereas the pattern of α-amylase was more or less similar in every part of the plant. This result is contrasting to that found in alfalfa taproots (Gana *et al.*, 1998) where the highest level of β-amylase genes was accmulated in the roots and stems instead of in the leaves. Whereas the result of the differential expression of α-amylase in all parts of the plant correlated with the physiology of cassava germination and root elongation in which the product from the starch degradation process (glucose) is used to support vegetative growth of the plants. The results suggested that both α-amylase and β-amylase might be involved in starch degradation of cassava at different developmental stages and, as well, in various processes.

Southern blot analysis was done using partial sequence of α-amylase and β-amylase as a probe. When the Southern blot was probed with α-amylase, the presence of a single band in the S*al*I and *Bam*HI treated samples indicated that α-amylase exists in the cassava genome as a single copy gene. When the Southern blot was probed with the β-amylase probe, two bands were observed in the *Bam*HI sample and three bands in X*ho*I samples. According to the restriction map of β-amylase, each endonuclease used in this experiment cut only once at one specific site. This data suggested that there is more than one copy of β-amylase in the cassava genome. Correlating with the cloning experiment, β-amylase is more abundant and are easily detected in cassava genome. Different patterns appeared after digestion

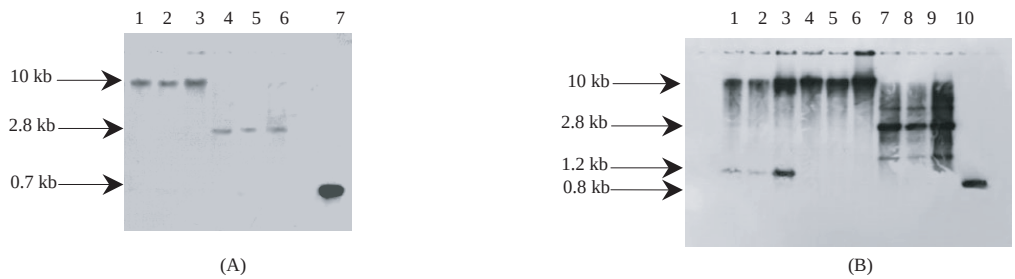

Figure 4 Southern hybridization of cassava genomic DNA. (A) Using α-amylase probe, Lane 1,2,3 cassava (HB60, KU50 and R1) DNA digested with *Sal*I and lane 4,5,6 digested with *Bam*HI, respectively. Lane 7 plasmid control digested with *Eco*RI. (B) Using β-amylase probe, Lane 1, 2, 3 cassava (HB60, KU50 and R1) DNA digested with *Xho*I, lane 4, 5, 6 digested with *Sal*I, lane 7,8,9 digested with *Bam*HI, respectively. Lane 10 plasmid control digested with *Eco*RI.

indicated different genes encoded both α-amylase and β-amylase.

CONCLUSIONS

Partial DNA sequence of α-amylase and β-amylase genes were identified and the expression was characterized in leaves, stems and storage roots. According to dot blot analysis and PCR studies, α-amylase and β-amylase might play an important role in starch degradation process, especially the expression of α-amylase in root. Further investigation of α-amylase and β-amylase expression at different stages of root development and vegetative growth would clarify their roles. The copy number determination of α-amylase and β-amylase genes, indicated that α-amylase exists in cassava genome as a single copy gene whereas β-amylase is identified as a multi-copied gene. The information obtained from this experiment including sequence, sequence analysis and mostly important gene expression, confirmed the complexity of starch degradation process in cassava.

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