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Differential Expression of Genes Involved in Sucrose Synthesis in Source and Sink Organs of Cassava Plants Undergoing Seasonal Drought Stress

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Abstract

Sucrose functions as a regulator of gene expression allowing plants to respond to cellular energy demands during both normal and stress conditions. Previous studies indicated that drought followed with onset of rain could negatively affect yield of starch in storage roots of cassava. In this study, molecular approaches were conducted to investigate expression profiling of sucrose phosphate synthase (SPS), sucrose phosphatase (SPP) and 14-3-3, which are importance as regulatory points to modulate availability of sucrose in cassava. In source organs, expression of cassava (*Manihot esculenta* Crantz.) sucrose phosphate synthase (*MeSPS*) and cassava sucrose phosphatase (*MeSPP*) varied depending on developmental stage and day/night cycle. In sink organ, the levels of *MeSPS*, *MeSPP* and *Me14-3-3* transcripts were affected to a much greater degree by different planting season than by root developmental stage. In response to fluctuating level of rainfall, a correlation between *MeSPS* and *Me14-3-3* expression patterns was observed. This finding suggests the role of these enzymes and the importance of sucrose metabolism in seasonal drought stress response in cassava. Given the identified new role for cassava as an important biofuel crop as well as its current usage as a food crop, understanding on the most appropriate time to plant and harvest cassava in order to maximize starch quantity and quality is warranted.

Keywords: cassava, sucrose phosphate synthase, sucrose phosphatase, 14-3-3 protein, storage root, seasonal drought

1. Introduction

Cassava (*Manihot esculenta* Crantz) is a perennial crop of major global importance, particularly in the developing world. The plant is grown for the underground tuberous storage roots, in which nearly 85% of its total dry weight is made up of starch (Tonukari, 2004). Compared with other starchy crops, cassava has relatively long growing cycle. Generally, it takes at least 7-8 months before storage roots could be harvested.

In Thailand, cassava is generally planted twice a year; in the beginning (from the end of February to the middle of June) and at the end of rainy season (from early October to early December), with its main growing area located in the eastern (i.e., Rayong province) and northeastern regions. Given that Thailand is located in the tropical zone, the average monthly temperature does not fluctuate widely throughout the year. This is in contrast with the average rainfall level, which could range from 72 mm to 1,418 mm during dry (November-April) and rainy (May-October) season respectively (data obtained from the Thai Meteorological Department, Ministry of Information and Communication Technology, Thailand). By observing that time and conditions at harvest had an impact on cassava starch properties, Sriroth et al. (1999) recommended that starch should be extracted from roots at either early or very late developmental stage. This was investigated further and the results indicated that changes in starch properties were mainly affected by drought, particularly when it was followed with the onset of rain (Santisopasri et al., 2001; Sriroth et al., 2001). To assure starch quality, Santisopasri et al. (2001) therefore

suggested that cassava should be planted at the start rather than at the end of rainy season. However, it is not yet clear how drought and water availability affect quality of cassava starch. To investigate into this matter, molecular approaches have been conducted to determine expression patterns of genes that might vary according to seasonal change in rainfall level.

When plants experience water stress, activation of sucrose phosphate synthase (SPS; EC 2.4.1.14), a key enzyme in sucrose synthesis, by reversible protein phosphorylation occurs, thus increasing the rate of sucrose synthesis (Quick et al., 1989). Sucrose-6-phosphate derived from this reaction is then cleaved by sucrose phosphatase (SPP, EC 3.1.3.24), which has been proposed to form a multi-protein complex with SPS (Echeverria et al., 1997). Moreover, the Ser-229 of spinach SPS has been shown to directly interact with 14-3-3 proteins in a phosphorylation and Mg^{2+}-dependent manner (Moorhead et al., 1999; Toroser et al., 1998). Although several studies indicated that binding of 14-3-3s generally inhibited SPS activity (Szopa et al., 2001; Toroser et al., 1998; Zuk et al., 2003), there was a report on partial activation of SPS after 14-3-3 binding (Moorhead et al., 1999).

Given the importance of SPS, SPP and 14-3-3 as regulatory points to modulate availability of sucrose, genes coding for SPS, SPP and 14-3-3 proteins have been cloned from various plants and experiments on protein-protein interaction have been carried out (Bornke, 2005; Castleden et al., 2004; Chen et al., 2005). However, none of these had been conducted in cassava. With promising future of cassava as an alternative biofuel crop (Nguyen et al., 2007), it would be essential to gain as much information as possible on the processes that underlie sucrose-to-starch transition and starch biosynthesis. As the first steps toward this goal, we isolated partial cDNA fragments encoding *MeSPS* and *MeSPP* and three full-length clones of *Me14-3-3*, namely *Me14-3-3; 1*, *Me14-3-3; 2* and *Me14-3-3; 3* cDNAs, from cassava. Subsequently, expression analyses by RNA gel blot and semi-quantitative RT-PCR were carried out to compare differential expression patterns of *MeSPS, MeSPP* and *Me14-3-3* in source and sink organs of plants grown in wet and dry seasons. As a result, it was observed that accumulation of *MeSPS, MeSPP* and *Me14-3-3* transcripts was affected to a certain degree by fluctuating level of rainfall. For cassava, changes in the apparent levels of *MeSPS*, *MeSPP* and *Me14-3-3* transcripts are probably considered essential for both growth and survival since they allow plants to respond to the cellular demand that might change according to the season.

2. Materials and Methods

2.1 Plant Materials

Cassava cultivar Kasetsart 50 (KU50) was grown in a field at Rayong Field Crops Research Center, Thailand in two different seasons. The dry crop was planted after the rainy season (in October), while the wet crop was planted during the rainy season (in May) of the following year. After 3, 6, 9 and 12 months of plantation, three plants were harvested and three storage roots were collected from each plant, washed, cut into small pieces and stored at -80°C. In addition to storage roots, leaves at two different developmental stages were collected, both during the day and at night. Young leaves were defined as the first unfolded leaves located at the top of each plant, while mature leaves were fully expanded with no visible sign of initial senescence.

2.2 RT-PCR Amplification of SPS, SPP and 14-3-3 cDNA Fragments

Total RNA (1 μg) extracted from frozen cassava tissues was used as template in first strand cDNA synthesis reactions and then in PCR to obtain partial cDNA fragments, namely *MepSPS*, *MepSPP* and *Mep14-3-3*. The primers used in PCR reactions were designed from consensus sequences of plant SPSs [SPS/1, CATGTG(CT)TGG(CA)G(GA)AT(TC)TGG; SPS/2, GCACCATCAACAAA(TC)TC(AC)GG], SPPs [SPP/1, CTCTTG(C/T)(C/T)TGTGG(T/C)GACTC; SPP/2, T(T/G)(C/T)TTCCC(T/A/C)T(G/T)TTTGTC(T/A)CCA] and 14-3-3s [14-3-3/1, C(C/G)ATTGA(G/A)CAGAAGGA(G/A)GA; 14-3-3/2, TCAGG(A/T/C)GAGTT (C/G/A)A(A/G)(A/G)ATCTC].

2.3 Construction and Screening of cDNA Libraries

Leaf and storage root cDNA libraries were constructed by inserting poly $(A)^+$ RNA into λTriplEx2 vector arms (SMART cDNA library construction kit, ClonTech). Hybridization was carried out at 65°C for 16-18 hrs in the presence of [^{32}P]-labeled *Mep14-3-3* DNA probe followed with washing under high stringency conditions (Church & Gilbert, 1984). By utilizing the *cre-lox* excision sites in λTriplEx2, pTriplEx2 vector carrying a cassava cDNA insert was obtained and sequenced.

2.4 Genomic Southern Blot

Aliquots of cassava genomic DNA (15 μg) were digested with restriction endonucleases (*Bam*HI, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Mlu*I, *Sac*I, *Sal*I and *Xba*I), fractionated on 0.8% agarose gels and blotted onto Hybond-N^+ (GE Healthcare Life Sciences). The blot was hybridized with *MepSPS, MepSPP* and *Me14-3-3;1*

cDNAs, labeled with digoxigenin (DIG)-dUTP (Roche). Hybridization was carried out at 68°C followed with washing at 65°C (Engler-Blum et al., 1993). After stripping, re-hybridization under lower stringency conditions (hybridization at 60°C followed with washing at 58 ºC) was performed.

2.5 Expression Analysis by RNA Gel Blot and Semi-quantitative RT-PCR

RNA gel blot analysis was carried out using 30 μg of total RNA extracted from leaf and storage root tissues of cassava. Hybridization was carried out for 16 hr at 65°C in the presence of *MepSPS, MepSPP* or *Me14-3-3;1* DNA probes, labeled with [^{32}P]-dCTP, followed with washing under high stringency conditions. Equal loading of RNA from various samples was determined by staining the membrane with methylene blue solution (0.04% methylene blue in 0.5 M sodium acetate; pH 5.2).

To perform semi-quantitative RT-PCR, 1 μl of cDNA template generated from 3-μg DNase-treated RNA samples was used as template in a 50-μl PCR reaction containing 0.4 μM of the gene-specific primers, 200 μM dNTPs and 1 unit *Taq* DNA polymerase (Fermentas). The gene-specific primers were SPS/3 (CATGTGTTGGAGGATT TGG), SPS/4 (GCACCATCAACAAATTCCG), SPP/3 (CTCTTGTTTGTGTGGTGACTCTG), SPP/4 (TTCTT CCCTTTTTTGTCTCCA), 14-3-3/3 (ATTGAGCAGAAGGAAGAG), 14-3-3/4 (ACGGTCAGGTGAGTTTAG), Actin/1 (CATGAGACTACATACAACTCCATC) and Actin/2 (TCGTACTCAGCCTTGGCAATCCAC). Subsequently, PCR was carried out using the conditions that had previously been optimized to allow the detection of *MeSPS*, *MeSPP* and *Me14-3-3* transcripts within the linear amplification phase (5 min at 95°C, 32 cycles of 1 min at 94°C, 1 min at 52°C (for *MeSPS* and *MeSPP*) or 48°C (for *Me14-3-3* and *MeActin*) and 2 min at 72°C, followed by 10 min at 72°C). The signals obtained from RNA gel blot and semi-quantitative RT-PCR were analyzed using Quantity One Software available in the Molecular Imager GS-800 calibrated densitometer (Bio-Rad).

3. Results

3.1 Cloning of cDNAs Encoding 14-3-3 Proteins in Cassava

The Arabidopsis genome contains up to 15 genes, 12 of which have been found to express and thus encode different GF14 isoforms. These GF14s contain a highly conserved core that is flanked by divergent termini. Degenerate primers designed from the core consensus sequences of Arabidopsis GF14s were used in RT-PCR to obtain a partial 14-3-3 cDNA from cassava. In the presence of leaf RNA templates, an amplified fragment of 373 bp, which showed significantly high degree of similarity to *14-3-3* mRNAs from plants, was obtained. To indicate that the resulting 373-bp cDNA encodes a partial *14-3-3* cDNA in cassava, the term *Mep14-3-3* cDNA was given and used throughout this study.

To gain information on the genes encoding 14-3-3 proteins in cassava, leaf and storage root cDNA libraries of cassava cv. KU50 were screened using *Mep14-3-3* cDNA, labeled with [^{32}P]-dCTP, as probe. Screening of 1.6 x 10^5 plaque-forming units of the leaf cDNA library yielded four positive clones, which differ from each other at the length of 5′ region. Therefore, only the clone that carries the longest cDNA insert, *Me14-3-3;1*, was further characterized (GenBank ID: DQ013887). By screening the storage root cDNA library, two full-length clones encoding 14-3-3 proteins, *Me14-3-3;2* and *Me14-3-3;3*, were identified (GenBank IDs: GQ922216 and GQ922217 respectively).

Me14-3-3;1, *Me14-3-3;2* and *Me14-3-3;3* cDNA are 1,186, 1,463 and 1,122 bp long, respectively (Figure 1). Although the length of these cDNAs is different, their coding regions are similarly composed of 795 nucleotides, thus coding for a polypeptide of 264 amino acids or 30 kDa. However, *Me14-3-3;1* cDNA shared higher degree of similarity to *Me14-3-3;3* cDNA (98% identity at nucleotide level and 95% identity at amino acid level) than to *Me14-3-3;2* cDNA (78% and 100% identity at nucleotide and amino acid level respectively). When compared with *grf* genes from Arabidopsis, the cassava cDNAs showed highest degree of similarity (84-90% identity) to *grf3* (GenBank ID: L09110), *grf7* (GenBank ID: U60445) and *grf5* (GenBank ID: L09109), respectively. To gain information on *Me14-3-3* encoding genes that might belong to other subgroups in the Arabidopsis phylogenetic tree, *Me14-3-3;1*, *Me14-3-3;2* or *Me14-3-3;3* were used to BLAST against the recently released cassava genome sequence database (http://www.phytozome.net/cassava). As a result, we identified 14 regions on the cassava genome that most likely contain *Me14-3-3*-encoding genes. Genes within these regions could give rise to major mRNA species of 16 different types, one of which possibly represents another *Me14-3-3* cDNA isolated from storage roots of cassava cv. KU50 (Sojikul et al., 2010). As expected, the polypeptides derived from these 16 mRNA species appeared to show high degree of similarity to those encoded by the Arabidopsis *grf* genes (Figure 2).

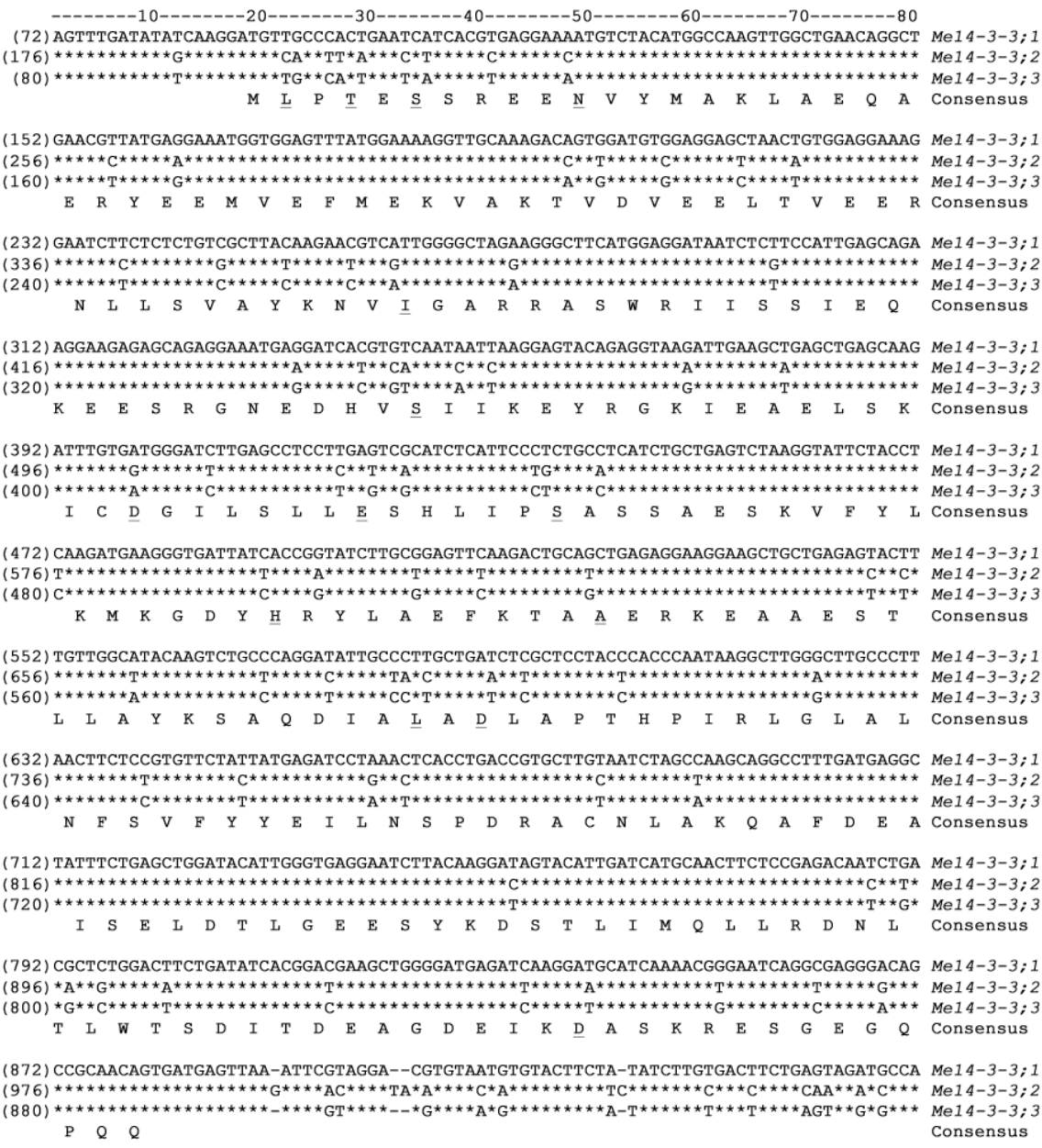

Figure 1. Alignment of *Me14-3-3;1* (GenBank ID: DQ013887), *Me14-3-3;2* (GenBank ID: GQ922216) and *Me14-3-3;3* (GenBank ID: GQ922217)

Amino acids deduced from these cDNAs were shown; however, at the positions where substitution occurs only the amino acids that appear as majority were indicated and underlined.

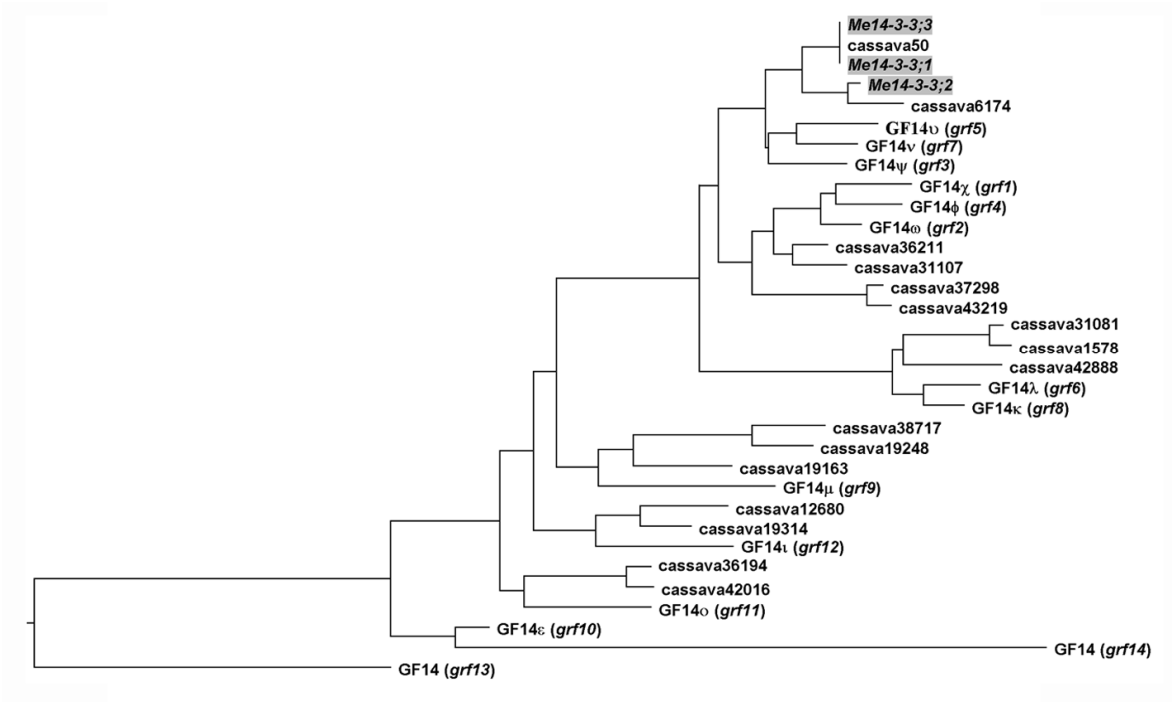

Figure 2. Phylogenetic relationship of 14-3-3 proteins constructed from deduced amino acid sequences of cassava and *Arabidopsis*

The deduced amino acid sequences are *Me14-3-3;1*, *Me14-3-3;2* and *Me14-3-3;3* cDNA (this study) and 16 different mRNA species from cassava obtained from the cassava genome sequence database, and also from 14 isoforms of GF14 from *Arabidopsis* (GF14χ, Swiss-Prot ID: P42643; GF14ω, Swiss-Prot ID: Q01525, GF14ψ, Swiss-Prot ID: P42644, GF14φ, Swiss-Prot ID: P46077; GF14υ, Swiss-Prot ID: AAB62225; GF14λ, Swiss-Prot ID: P48349; GF14ν, Swiss-Prot ID: AAD51782, GF14κ, Swiss-Prot ID: AAD51783; GF14μ, Swiss-Prot ID: AAD51784; GF14ε, Swiss-Prot ID: P48347; GF14ο, Swiss-Prot ID: AAG47840; GF14ι, Swiss-Prot ID: AAF98570. Three remaining isoforms of *Arabidopsis* GF14, which appear under Swiss-Prot IDs: AAG52105, AAF87262 and AAD28654, are currently designated as 14-3-3 like proteins since expression of the genes that encode them have not yet been observed. The *grf* gene encoding each of these GF14s are specified in brackets.

3.2 Cloning of Partial cDNAs Encoding SPS and SPP in Cassava

Partial cDNAs encoding *MeSPS* and *MeSPP* were obtained by RT-PCR using cassava leaf RNA as template. Amplification of *MepSPS* cDNA was carried out using primers designed from consensus sequences of the SPS N-terminal glycosyltransferase domain. The amino acid sequence deduced from the *MepSPS* cDNA showed 85% and 73% identity to the two isoforms of Arabidopsis SPS family A encoded by *AtSPS1F* (GenBank ID: NM_122035) and *AtSPS2F* (GenBank ID: NM_121149), while it shared only 63% and 54% identity to the family B (*AtSPS3F*; GenBank ID: NM_100370) and family C isoforms (*AtSPS4F*; GenBank IDs: NM_001036532 and NM_117030), respectively. These results therefore suggest that the 621-bp *MepSPS* cDNA is derived from the gene encoding SPS family A in cassava. In addition to the glycosyltransferase domain, *MepSPS* cDNA also encodes two serine residues, whose positions correspond to the Ser-158 and Ser-229 of spinach SPS (Figure 3).

To obtain *MepSPP* cDNA, RT-PCR was carried out using forward and reverse primers derived from consensus sequences within the N-terminal L-2-haloacid dehalogenase (HAD) domain and the C-terminal extension region of plant SPPs, respectively.

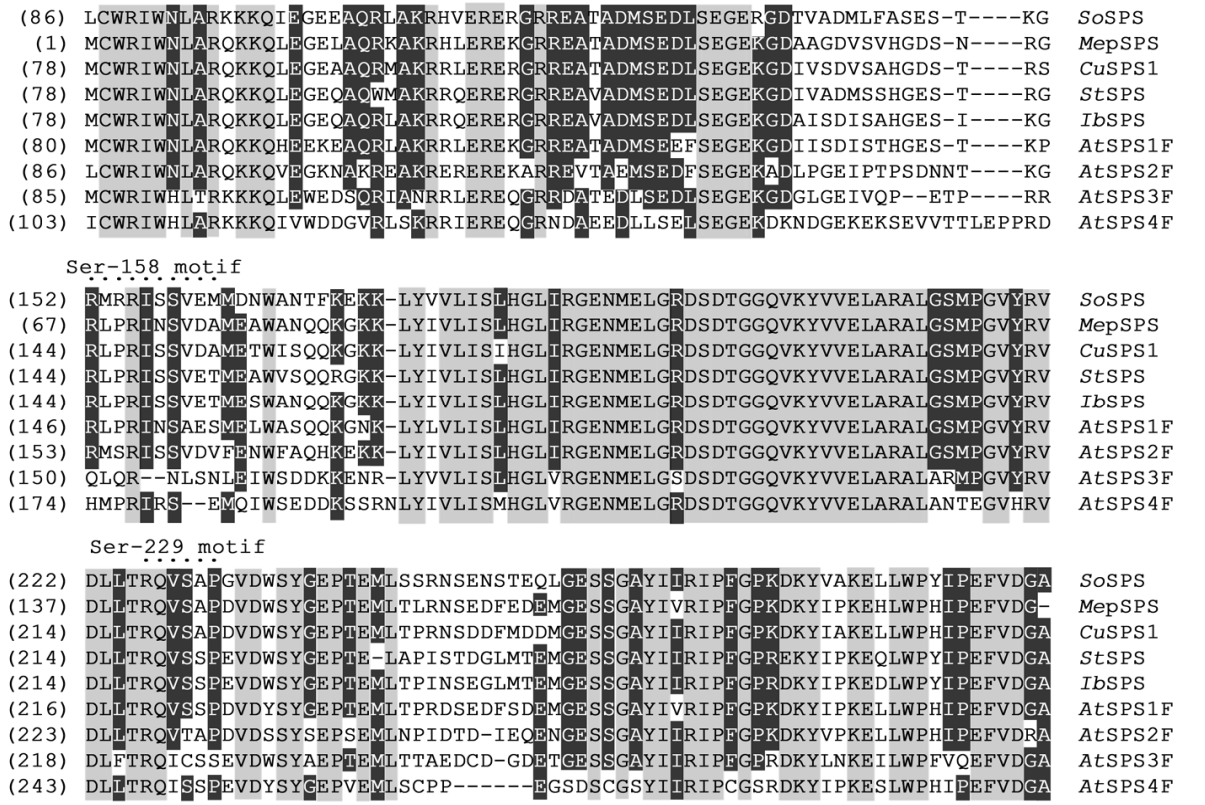

Figure 3. Alignment of amino acid sequences encoded by 621-bp of *SPS* cDNA from cassava, spinach, citrus, potato, sweet potato and *Arabidopsis*

The amino acid sequences encoded by 621-bp *MepSPS* cDNA (GenBank ID: GQ922214), *SoSPS* mRNA from *Spinacia oleracea* (GenBank ID: L04803), *CuSPS1* mRNA from *Citrus unshiu* (GenBank ID: AB005023), *StSPS* from *Solanum tuberosum* (GenBank ID: X73477), *IbSPS* mRNA from *Ipomoea batatas* (GenBank ID: AF439861) and *Arabidopsis thaliana* mRNAs designated as *AtSPS1F* (GenBank ID: NM_122035), *AtSPS2F* (GenBank ID: NM_121149), *AtSPS3F* (GenBank ID: NM_100370) and *AtSPS4F* mRNAs (GenBank ID: NM_117080). Residues conserved in all and most of the sequences are shown in gray and black, respectively. The residues that are included in the Ser-158 and Ser-229 motifs are indicated by dots above.

3.3 DNA Gel Blot Analysis of Genes Encoding MeSPS, MeSPP and Me14-3-3

Based on the allopolyploid nature of cassava, the number of bands, observed by DNA gel blot, is not necessarily equal copy number of genes present in the genome. In our case, interpretation of the results from DNA gel blot analysis of genes encoding *Me*SPS, *Me*SPP and *Me*14-3-3 is even more complicated due to insufficient information on the genomic sequences. As a result, multiple banding patterns observed by DNA gel blot might derive from different regions of the same gene. To minimize this problem, restriction maps of genes from various plant species were determined and only the enzymes that cannot cut within the genes were chosen for this study. When using *MepSPS* cDNA as probe, multiple digested products could be observed (Figure 4a). The result has therefore suggested that multiple copies of *MeSPS* gene are present in the cassava genome. Based on the multiple banding patterns detected by the *MepSPP* cDNA probe (Figure 4b), *MeSPP* should also exist as multiple copy genes in the cassava genome.

Unlike *MeSPS* and *MeSPP*, DNA gel blot analysis using *Me14-3-3;1* cDNA probe could result in the detection of only a few fragments (Figure 4c). To determine that the genes of significantly lower similarity might exist in the cassava genome, the probe was striped and hybridization at low stringency condition was performed. According to Figure 4d, several additional fragments were observed, thus indicating that multiple copies of *Me14-3-3* genes exist in the cassava genome.

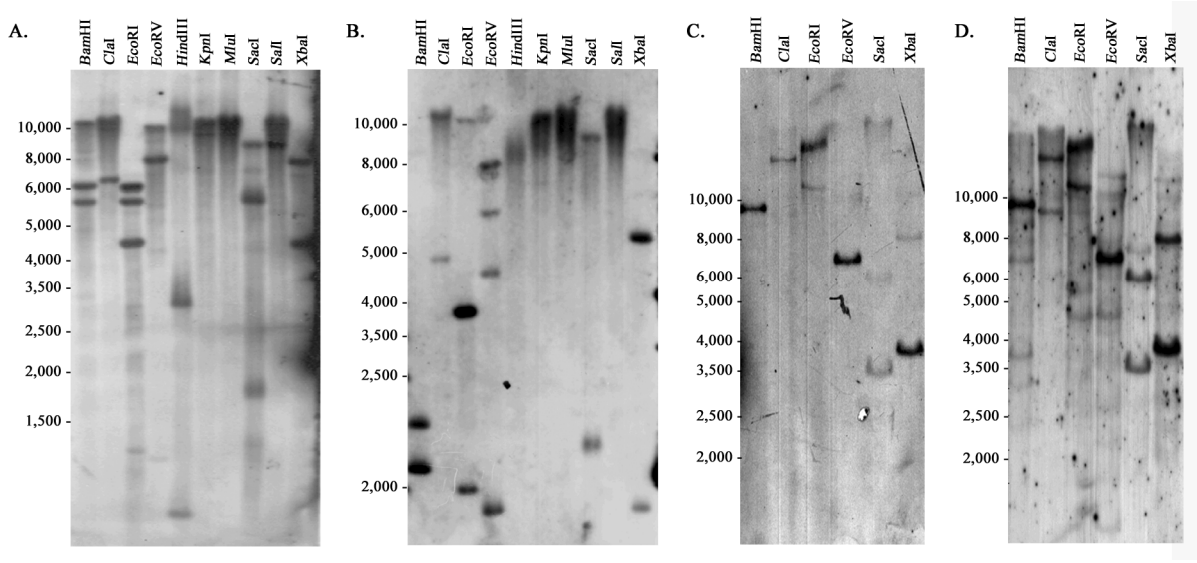

Figure 4. DNA gel blot analysis probed with MepSPS (A), MepSPP (B) and Me14-3-3;1 (C and D carried out under high and low stringency conditions, respectively)

The sizes in base pairs of the DNA molecular weight bands are indicated on the left.

3.4 Differential Expression Patterns of MeSPS, MeSPP and Me14-3-3

RNA gel blot was employed to investigate temporal expression patterns of *MeSPS, MeSPP and Me14-3-3* in storage roots of cassava planted in wet and dry season. According to Figure 5, different sized transcripts of 3.5 and 2.0 kb were detected by *MepSPS* cDNA probe. Based on the size of other plant *SPS* mRNAs, the 3.5-kb transcript would most likely represent full-length *MeSPS* mRNA, while the presence of smaller sized transcript has not been observed elsewhere. Therefore, it remains to be investigated whether the 2.0-kb *MeSPS* mRNA arises from partial degradation of the large transcript, from alternative splicing of the single pre-mRNA, or, alternatively, from different genes. No matter what would be the cause for the presence of two different sized *MeSPS* transcripts, the results shown in Figure 4 indicated that both transcripts had comparatively similar expression patterns and their levels in cassava storage roots also followed each other closely. However, we observed that the signals obtained from RNA gel blot were considered fairly low even from 30 μg of total RNA as starting material. Alternatively, semi-quantitative RT-PCR was carried out using the conditions (i.e. cycle number and concentrations of primers, template and $MgCl_2$) that result in the detection of *MeSPS,* and also *MeSPP* and *Me14-3-3*, in the linear amplification phase. Along with cDNAs, RNA samples pre-treated with DNaseI were also used as templates in RT-PCR. This was to ensure that the RNA samples used in first strand cDNA synthesis reactions were devoid of genomic DNA contamination.

Results obtained from RNA gel blot (Figure 5) and semi-quantitative RT-PCR (Figure 6) similarly indicated that the levels of *MeSPS* transcripts increased as the roots of dry crop developed from 3 to 6 and 9 months old. As the plants matured further, the levels of *MeSPS* mRNAs decreased gradually. For plants grown in the wet season, abundant levels of *MeSPS* transcripts were present in storage roots at 3 months old and then decreased to significantly low levels at later stages.

Unlike *MeSPS*, the levels of *MeSPP* transcript fluctuated only slightly during the course of storage root development (Figure 5). When growing season was taken into account, we observed that *MeSPP* transcript was present at slightly higher level in plants grown in the wet season (Figures 5 and 6). Although this finding was observed by both RNA gel blot and semi-quantitative RT-PCR, the high sensitivity of PCR allowed the result to be more easily detected.

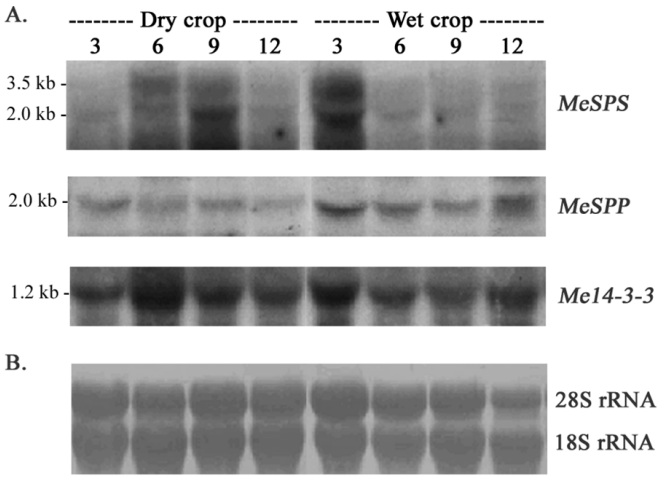

Figure 5. Temporal expression patterns of *MeSPS, MeSPP* and *Me14-3-3* in storage roots of cassava

The cassava plants were grown for 3, 6, 9 and 12 months in wet and dry season. RNA gel blot analysis was carried out by priming 30 μg of total RNA with *MeSPS, MeSPP* or *Me14-3-3;1*, labeled with [^{32}P]-dCTP, as probes (A). Equal loading of RNA from various samples was determined by staining the membrane with methylene blue solution (B).

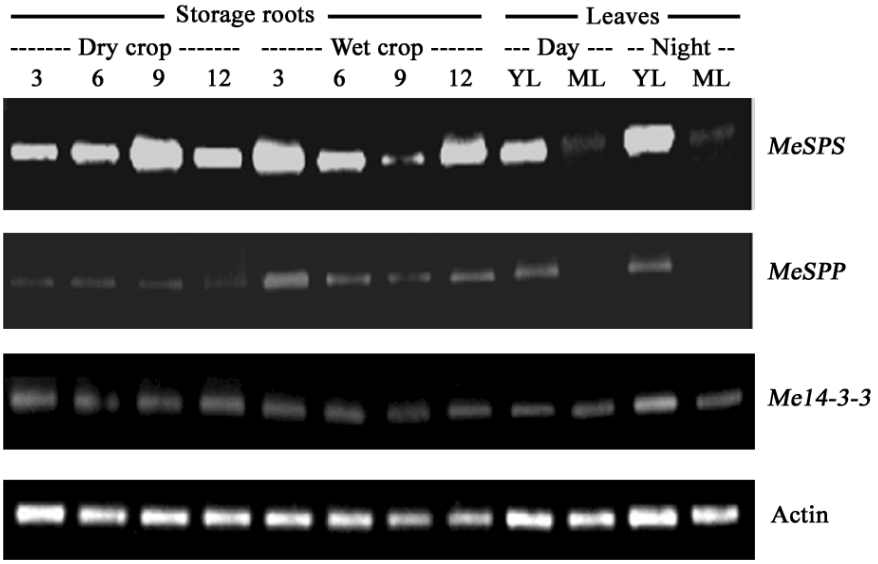

Figure 6. Expression profiles of *MeSPS, MeSPP* and *Me14-3-3* in storage roots of cassava

The cassava plants were grown for 3, 6, 9 and 12 months in wet and dry season and in young (YL) and mature (ML) leaves collected during the day or at night observed by semi-quantitative RT-PCR using actin as an internal control

Expression analysis of *Me14-3-3* by RNA gel blot resulted in the detection of 1.2-kb transcript in storage roots of both wet and dry crops (Figure 5). Although the levels of 1.2-kb transcript seemed to rarely fluctuate, it was nonetheless possible to indicate that accumulation of *Me14-3-3* transcript was present at slightly higher level in dry crop roots at 6 months old and in wet crop roots at 3 months old. Based on the size of *Me14-3-3* cDNAs (Figure 1), slight variation in 1.2-kb transcript level could only reflect the accumulation of *Me14-3-3;1* and *Me14-3-3;3* transcripts in storage root tissue of cassava. Unlike results obtained from RNA gel blot, semi-quantitative RT-PCR indicated that *Me14-3-3* was expressed at relatively stable levels in all cassava tissues

tested in this study (Figure 6). Since the primers used in semi-quantitative RT-PCR were designed from the core consensus sequence of *Me14-3-3* cDNAs, the results obtained did not allow us to identify which of the *Me*14-3-3 encoding genes that might be differentially expressed during the course of cassava root development. Given that this is the first report on *Me14-3-3*s from cassava and the three *Me14-3-3* mRNAs showed extremely high degree of similarity to each other (78-98% identity at nucleotide level and 98-100% identity at amino acid level), it would be very difficult to obtain primers that are specific to each *Me14-3-3* encoding gene. Based on the results obtained, however, it is possible to indicate that temporal expression patterns of *Me14-3-3*, particularly those derived from the genes encoding *Me14-3-3;1* and *Me14-3-3;3*, vary depending on the root developmental stage and different planting season.

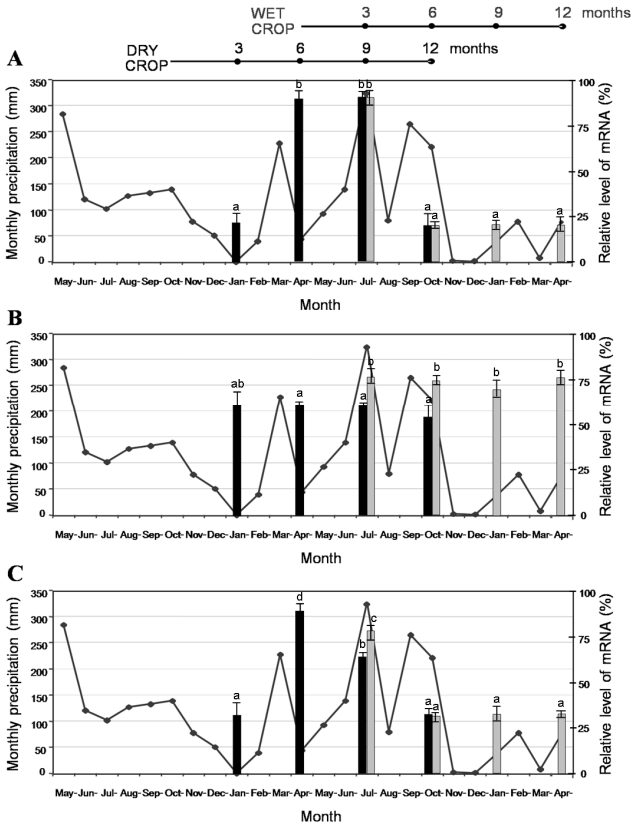

Figure 7. Accumulation of *MeSPS* (A)*, MeSPP* (B) and *Me14-3-3* (C) gene transcripts in response to fluctuating levels of rainfall, dry crop (black) and wet crop (grey)

RNA gel blot analysis was carried out using 30 μg of total RNA extracted from 3 storage root tissues obtained from 1 cassava plant. The 3 independent replicate experiments were performed. Intensity of the signals observed by RNA gel blot analyses was analyzed by QuantityOne software (Bio-rad) and plotted vs. monthly precipitation levels. Data correspond to the mean of 3 independent replicate experiments (n=3), the error bars represents the standard error of the mean (SE). The significant differences were determined by DNMRT using the SPSS software package. Values followed by the same letter are not significantly different ($p < 0.01$) using DNMRT.

To obtain information on *MeSPS, MeSPP* and *Me14-3-3* expression patterns in source tissues, cassava leaves at different developmental stages (young and mature) and carbon partitioning status (harvested during the day and at night) were utilized as starting materials for semi-quantitative RT-PCR analysis (Figure 6). In young leaves, abundant level of *MeSPS* transcript was observed and its level seemed to be slightly higher in leaves harvested at night. In mature leaves, a significantly low level of *MeSPS* mRNA was observed and its levels did not seem to be affected by the day/night cycle. For *MeSPP*, accumulation of the transcript was observed only in young leaves and its levels in leaves harvested at different time of day were comparatively stable. Similar to the results observed in storage root tissue, the signals derived from *Me14-3-3* transcripts appeared to be unchanged (Figure

6). Again, this possibly resulted from the inability of the primers to distinguish between each of the *Me14-3-3* cDNA template. Accordingly, stable accumulation of the 375-bp RT-PCR product would reflect ubiquitous existence of 14-3-3 proteins in cassava tissues rather to indicate that all the 14-3-3-encoding genes are stably expressed in all types of cassava tissues. Accumulation of *MeSPS, MeSPP* and *Me14-3-3* transcripts in response to fluctuating levels of rainfall, dry crop and wet crop were determined (Figure 7). And the *cis*-acting elements in the 5′-upstream region of *MeSPS, MeSPP* and *Me14-3-3* genes were also analyzed (Figure 8).

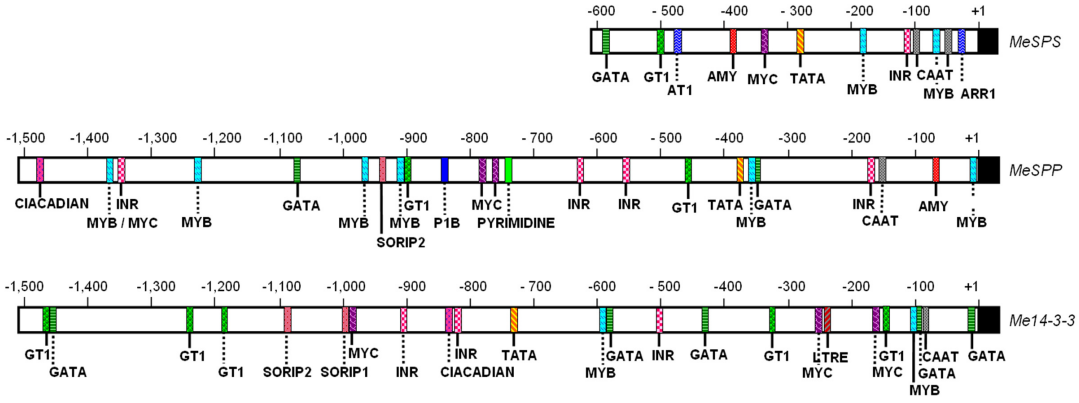

Figure 8. Diagrammatic illustration of various *cis*-acting elements in the 5′-upstream region of *MeSPS, MeSPP* and *Me14-3-3* genes

Cis-acting elements in the 5′-upstream region were analyzed by PLACE software (http://www.dna.affrc.go.jp/PLACE/). Positions of the *cis*-acting elements, including dehydration responsive elements (*i.e.* Myb and Myc binding sites) and a drought responsive LTRE box, are indicated with negative numbers in relation to the translational start site at +1 position.

4. Discussion

The 14-3-3s are ubiquitous proteins that have been observed in virtually every eukaryotic organism. According to Rosenquist et al. (2001), the Arabidopsis genome contains up to 15 genes (*grf1* to *grf15*), 12 of which have been found to express and thus encode different GF14 isoforms designated as chi (χ), omega (ω), psi (ψ), phi (ϕ), upsilon (υ), lambda (λ), nu (ν), kappa (κ), mu (μ), epsilon (ε), omicron (ο) and iota (ι). These GF14s contain a highly conserved core that is flanked by divergent termini. In addition to Arabidopsis, other plants also possess multiple isoforms of 14-3-3 protein, each of which is encoded by highly conserved yet distinct gene (Konagaya et al., 2004; Wu et al., 1997; Xu et al., 2006). In this study, three full-length cDNAs encoding 14-3-3 proteins were isolated from cDNA libraries of cassava. The *Me14-3-3;1* cDNA, which was isolated from the leaf cDNA library, is 1,186 bp long, while the *Me14-3-3;2* and *Me14-3-3;3* cDNAs obtained from the storage root cDNA library were 1,463 and 1,122 bp in length, respectively. Although the length of these cDNA clones is different, they similarly encode a polypeptide of 264 amino acids or 30 kDa. Both nucleotide and deduced amino acid sequences of *Me14-3-3;1* cDNA are more closely related to those of *Me14-3-3;3* than to *Me14-3-3;2* cDNA. According to Rosenquist et al. (2001), the Arabidopsis *grf1* to *grf14* genes have been divided into two major branches in the phylogenetic tree. The first branch is composed of three subgroups; *grf1*/*grf2*/*grf4*, *grf3*/*grf5*/*grf7* and *grf6*/*grf8*. The other branch includes the remaining *grf9* to *grf14*. *Me14-3-3* cDNAs showed highest degree of similarity (85-90%) to members in the *grf3*/*grf5*/*grf7* subgroup, and then to *grf1*/*grf2*/*grf4* (79-83%) and *grf6*/*grf8* (72-74%), respectively. According to Sehnke et al. (2000), the products of *grf7* and *grf5* are among two of the four GF14s that were identified to be located inside chloroplast stroma, thus suggesting their specialized role in metabolic pathways of chloroplasts.

When compared with 17 isoforms of tobacco 14-3-3 proteins (Konagaya et al., 2004), the proteins encoded by *Me14-3-3* cDNAs showed highest similarity to the tobacco isoform e1 and e2. These tobacco isoforms have been shown to have intermediate binding affinity with SPS (Bornke, 2005). This corresponds to the results observed in spinach (*Spinacia oleracea*), where direct binding of 14-3-3 to the phosphorylated Ser-229 of *So*SPS has been indicated (Toroser et al., 1998). By conducting RT-PCR using degenerate primers designed from consensus

sequences of plant SPSs, a 621-bp *MepSPS* cDNA was obtained. Deduced amino acid sequence of the *MepSPS* cDNA indicate that it encodes a serine residue that corresponds to the Ser-229 of *So*SPS. Similar to SPSs from various plant species, the 'Ser-229' of *MepSPS* is also located within the consensus motif R-X-X-S*-X-P (where S* indicates the phosphorylated serine). The presence of this motif in *Me*SPS probably suggested that interaction between *Me*SPS and *Me*14-3-3(s) was also conserved in cassava.

Partial cDNAs encoding *MeSPS* and *MeSPP* were obtained by RT-PCR using cassava leaf RNA as template. Amplification of *MepSPS* cDNA was carried out using primers designed from consensus sequences of the SPS N-terminal glycosyltransferase domain. According to BLAST (Mauchler-Bauer et al., 2009), the glycosyltransferase domain encoded by 621-bp *MepSPS* (GenBank ID: GQ922214) could be further classified as GT1 family, which is exclusively observed in enzymes from plants or photosynthetic bacteria. Phylogenetic analysis of plant SPSs has classified the enzymes into three families, designated as A, B and C, with monocot/dicot diversions present in each family (Langenkamper et al., 2002; Lutfiyya et al., 2007). The amino acid sequence deduced from the *MepSPS* cDNA showed 85% and 73% identity to the two isoforms of Arabidopsis SPS family A encoded by *AtSPS1F* (GenBank ID: NM_122035) and *AtSPS2F* (GenBank ID: NM_121149), while it shared only 63% and 54% identity to the family B (*AtSPS3F*; GenBank ID: NM_100370) and family C isoforms (*AtSPS4F*; GenBank IDs: NM_001036532 and NM_117030), respectively. These results therefore suggest that the 621-bp *MepSPS* cDNA is derived from the gene encoding SPS family A in cassava.

The glycosyltransferase domain, *MepSPS* cDNA is found to encode two serine residues, whose positions correspond to the Ser-158 and Ser-229 of spinach SPS. Phosphorylation of the spinach Ser-158 and Ser-229 has been indicated to involve in light/dark modulation and 14-3-3 protein binding, respectively (Huber & Huber, 1992; Toroser et al., 1998). Similar to the Ser-158 and Ser-229 of spinach leaf SPS, the corresponding residues of cassava SPS were observed within the consensus motif B-Hy-X-B-X-X-S*-X-X-Hy (where B is a basic residue, Hy a hydrophobic residue, S* the phosphorylated serine and X any amino acid) (Huang & Huber, 2001), and R-X-X-S*-X-P, respectively (Winter & Huber, 2000).

The *MepSPP* cDNA was obtained using primers derived from consensus sequences within the HAD domain and the C-terminal extension region of plant SPPs respectively. It is important to note that in addition to the glycosyltransferase domain, plant SPSs also contain an SPP-like domain, which shows some similarity (35%) to the catalytic HAD domain of SPP (Lunn et al., 2000). By including the fragment that codes for the C-terminal extension region of SPP into the RT-PCR product, non-specific amplification of *MeSPS* cDNA could therefore be minimized. As expected, the 460-bp RT-PCR product (GenBank ID: GQ922215) not only showed high degree of similarity to *SPP* mRNAs but encodes a part of the catalytic HAD phosphatase domain containing a part of the third, from a total of three, highly conserved motifs typical of enzymes in the HAD superfamily (Aravind et al., 1998).

When growing season was taken into account, it could be observed that the dry crop was planted when the rainfall level was low and this condition continued until the first onset of rain in the 5th month. At the onset of rain in the 6th month, growth resumes using the energy derived from degradation of reserve starch (Pardales & Esquibel, 1996; Sriroth et al., 2001). At this stage, the net flux of sucrose metabolism would be toward the rate of sucrose (re)synthesis, thus explaining the increase in *MeSPS* transcript in the roots of dry crop harvested at 6 months old. Since the conditions that resemble drought followed with the onset of rain still persisted from 6th to 9th month, high level of *MeSPS* expression could still be observed at 9 months old. With the return of drought in the following year, marked decrease in *MeSPS* transcript level was observed. This finding was similarly observed in both wet and dry crop, thus indicating that plants grown in different season responded to drought in a similar manner. In growing potato tubers, short-term water deficit was found to activate *SPS*, thus leading to a switch from net sucrose degradation to net sucrose synthesis (Geigenberger et al., 1997 & 1999). Although our study in cassava produced conflicting results, these studies similarly indicated that differential expression of *SPS* was essential in water stress response in plants. However, the mechanisms underlying drought-stress responses vary depending on plant species. The change in rainfall level seemed to affect *Me14-3-3* expression in the same way as it did to *MeSPS*. The relatively similar patterns of *MeSPS* and *Me14-3-3* might indicate that these proteins need to co-exist to form a multi-protein complex. However, it is important to note that the results most likely reflected only the levels of *Me14-3-3;1* and *Me14-3-3;3* mRNA but not *Me14-3-3;2* transcript.

To support the idea that expression of *MeSPS, MeSPP* and *Me14-3-3* could be triggered by seasonal drought stress, 5′-upstream regions of these putative genes were downloaded from cassava genomic sequences in Phytozome database (https://www.phytozome.net/cassava) and analyzed for putative *cis*-acting elements using PLACE software (http://www.dna.affrc.go.jp/PLACE/) (Higo et al., 1999). As a result, several dehydration responsive elements such as Myb and Myc binding sites as well as a drought-responsive LTRE box (Baker et al.,

1994) were observed. These results therefore suggest that expression of *MeSPS* and *Me14-3-3* genes are influenced by changes in environmental conditions such as drought. In addition, both *MeSPS* and *MeSPP* promoters also contain an alpha-amylase (Amy) responsive element (Huang et al., 1990).

By conducting RNA gel blot analysis, we observed two different sized *MeSPS* transcripts, 3.5 and 2.0 kb in length, in storage root tissue of cassava. However, the results clearly indicated that both transcripts had comparatively similar expression patterns and their levels in cassava storage roots also followed each other closely. Similarly, multiple SPS transcripts were observed in wheat (Castleden et al., 2004) and banana (do Nascimento et al., 1997). However, identity of the truncated transcripts is still poorly understood. For cassava, it remains to be investigated whether the truncated *MeSPS* mRNA arises from partial degradation of the 3.5-kb transcript, from alternative splicing of the single pre-mRNA, or, alternatively, from different genes. Based on the results obtained from DNA gel blot analysis, it seems possible that these transcripts might derive from different genes located in the cassava genome.

In response to fluctuating level of rainfall, a correlation between *MeSPS* and *Me14-3-3* expression patterns was observed. This finding not only supports the idea that these proteins need to co-exist to form a multi-protein complex, but also suggests that the apparent levels of *MeSPS* and *Me14-3-3* transcripts need to be tightly regulated. This type of regulation is supposedly important for both growth and survival of cassava since it allows plants to respond to the cellular energy demand that might change according to the season. In contrast to *MeSPS* and *Me14-3-3*, relatively stable levels of *MeSPP* transcript were observed during storage root development. However, when RT-PCR was carried out, it was observed that the levels of *MeSPP* transcript fluctuated slightly, particularly in storage roots of wet crop harvested at 3 months old. We therefore hypothesize that the high levels of *MeSPP* and *MeSPS* transcripts observed at this stage possibly reflects the increasing rate of sucrose (re)synthesis to supply energy derived from degradation of reserve starch.

In source organs, we observed high expression level of *MeSPS* in young leaves and significantly lower level in mature leaves. These results were inconsistent with *Actinidia chinensis*, in which no significant changes in *AcSPS* transcript level could be observed in leaves at different developmental stages (small, expanding and senescence leaves) (Fung et al., 2003). These probably result from, for example, the diverse nature of plants, the different turnover rate of leaf sucrose and the different criteria used to identify the leaf developmental stage. Additionally, we also observed that expression of *MeSPS* in cassava leaves varied depending on the day/night cycle. This was particularly true for young leaves, in which a slightly higher level of *MeSPS* transcript was observed in leaves harvested at night. At night, leaf starch is degraded to form sucrose for storage in the vacuole and/or for transport through phloem to storage organs. During this stage, the rate of sucrose synthesis increases and this therefore explains why a slightly high level of *MeSPS* transcript could be observed. For *MeSPP*, high level of the leaf transcript was observed in young leaves, while none was detected in mature leaves. In comparison with the levels of *MeSPS* transcript, *MeSPP* was always present at a significantly lower level. Therefore, it was not surprising to detect none of the *MeSPP* transcript in mature cassava leaves, in which *MeSPS* was present at relatively low level.

5. Conclusion

Previous studies by Pardales et al. (1996) and Sriroth et al. (2001) indicated that drought followed with onset of rain could negatively affect yield of starch in storage roots of cassava. To gain more insight into the metabolic fate of starch, the investigation of expression profiling of the three genes involved in the cassava sucrose synthesis was conducted. In conclusion, the results from this study indicated that the fluctuating levels of *MeSPS, MeSPP* and *Me14-3-3* transcripts in sink organ, seemed to be affected to a much greater degree by different planting season than by developmental stage of the roots. Although various factors could contribute to the difference in planting season, it seems that the amount of water available to the plants has played a significant role in determining expression patterns of *MeSPS, MeSPP* and *Me14-3-3* in storage root tissue of cassava. Given that metabolic processes are generally regulated at multiple levels, further studies need to be conducted to provide more information on *MeSPS, MeSPP* and *Me14-3-3* isoforms, as well as their regulation at both transcriptional and post-transcriptional levels. The knowledge obtained should lead us to understand the role of these enzymes and the importance of sucrose metabolism in seasonal drought stress response in cassava. Due to the identified new role for cassava as a possible biofuel crop as well as its current usage as a food crop, further understanding on the correct time to plant and harvest cassava in order to maximize starch quantity and quality is warranted.

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