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Isolation and Characterization of MADS Box Genes Possibly Related to Root Development in Sweetpotato (*Ipomoea batatas* L. Lam.)

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Development of the tuberous root of sweetpotato coincides with abnormal vascular cambium activity. The genes and pathways predominately active for vascular morphogenesis in sweetpotato root are unknown. In this study, using a reverse transcription-polymerase chain reaction (RT-PCR) approach, we found three sweetpotato MADS box genes expressed in root tissues. *IbAGL17* is an *AGL17* clade MADS box gene, and was mainly expressed in tuberous root as demonstrated by RT-PCR analysis. *IbAGL20* is a *TM3*-like gene that is expressed at similar levels in leaf, vegetative shoot, flower and root tissues. *IbMADS79* closely resembled the *Arabidopsis AP1* and *Antirrhinum SQUA* genes, but *IbMADS79* was expressed only in roots, whereas the mRNA levels of genes of *SQUA* clade were high in the flower and floral meristem. RT-PCR results confirmed that MADS box genes were also expressed in different developmental stages. These differentially expressed MADS box genes will be potential candidates for research to elucidate the molecular process related to root development in sweetpotato.

Keywords: IbMADS79 clade, MADS box gene, root development, sweetpotato

Sweetpotato (*Ipomoea batatas* L. Lam.) is an economically important crop, and also an interesting plant because it can propagate from vegetative tissues, root tubers and vine cuttings, as well as from seed (Onwueme, 1978). The tuberization process in sweetpotato and potato is known to be controlled by environmental factors, such as photoperiod, temperature, oxygen concentration, moisture and soil properties (Onwueme, 1978). The processes regulating the tuberization from fibrous root to tuberous root growth is very important for a tuber crop, like sweetpotato. Especially, sweetpotato has a specific root organ system, and breeding programs is aimed at favorable traits related to storage root production. The genes governing the root development are poorly characterized and remain unknown in tuber crops. Investigation of the underlying changes associated with root organogenesis will be useful for the study of morphogenesis and for genetic improvement of plants.

It is very likely that at least some of the genes controlling the tuberization are members of the MADS box gene family. MADS box genes are mostly regulators of developmental processes (Shore and Sharrocks, 1995). Recently, some genes in *Arabidopsis*, such as *AGL12*, *AGL19*, *AGL17* and *ANR1*, seem to play a role in vegetative tissue development (Rounsley et al., 1995; Zhang and Forde, 1998). Interestingly, *ANR1* function is related to the development of lateral roots in response to nitrate availability within the soil, thus linking environmental conditions and vegetative development (Zhang et al., 1999). In addition, the alfalfa genes *NMHC5* and *NMHC7* (Heard et al., 1997) are expressed in root nodules, which are root-derived structures induced upon symbiotic association with *Rhizobium* bacteria, indicating the participation of MADS box genes in developmental programs triggered by external signals (Garcia-Maroto et al., 2000; Kang and Kang, 2002). Recently, the *AGL17* subfamily continues to grow, and while these classes of MADS box genes are largely vegetative in nature, we believe them to be the primary classes of MADS box genes that hold important keys to the development of whole plants including roots, stems, leaves, and plant vascular system. It is important to note that MADS box genes are currently known to have functions in vegetative development.

The three genes resemble the *SQUA*-like genes in the *Arabidopsis* genome, *APETALA 1* (*AP1*), *CAULIFLOWER* (*CAL*), and *AGL8* (Mandel et al., 1992; Kempin et al., 1995; Gu et al., 1998). The first *SQUA*-like gene cloned was the floral meristem identify gene *SQUAMOSA* (*SQUA*) from *Antirrhinum* (Huijser et al., 1992). Many *SQUA*-like genes are typically expressed in the inflorescence or floral meristems, and, accordingly, most of them may work as meristem identifying genes (Theißen

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et al., 1996). In addition, *AP1* is involved in specifying sepals and petals and thus works as a class a floral organ identity gene (Mandel et al., 1992). Interestingly, *AGL79* is a newly identified *SQUA* homolog with unknown function but found to be expressed in roots (Parenicova et al., 2003) providing evidence that a new class of MADS-box genes with *IbMADS79* of sweetpotato might be involved in the regulatory network directing vegetative organogenesis during plant growth.

The *TM3*-class MADS box genes are root specific and includes the tomato *TM3* gene which is abundant in leaves and early floral meristems (Pnueli et al., 1992). Recent work on *Arabidopsis* has identified several *TM3* class transcription factors, *SOC1/AGL20* (Onouchi et al., 2000; Samach et al., 2000), *AGL14*, *AGL19* (Rounsley et al., 1995), *AGL42*, *AGL71* and *AGL72* (Parenicova et al., 2003). *SOC1/AGL20* is involved in the mechanisms required to promote flowering including those influenced by vernalization, photoperiod, and other autonomous pathways (Lee et al., 2000).

In this study, in order to isolate MADS box genes that may be related to root development in sweetpotato, we performed RT-PCR analysis with degenerate MADS box primers using the developing tuberous root of sweetpotato. One of the isolated genes bears resemblance to the *AGL8*, *AP1* of *Arabidopsis* and *SQUA* of *Antirrhinum*, which play a role in floral transition. The other two genes are involved in *AGL17* clade and *TM3* clade.

MATERIALS AND METHODS

Plant Materials

Sweetpotato (*I. batatas* L. cv. Kokei 14) grown in the field were harvested to analyze MADS box genes in different organs of the adult plants.

Isolation of MADS Box Genes from Developing Tuberous Root

The CTAB method (Kim et al., 2002a) was revised for extraction of RNA from sweetpotato. Conserved sequence between MADS box genes are generated using GENETYX (Software Development, Japan). One degenerated primer was designed according to the conserved amino acid sequence QVTFSKR [5′-GIA (A/G)GTIACITT(C/T)(A/T)(G/C)IAA(C/T)(A/T)G-3′] in the MADS-box an alignment. First strand cDNA was synthesized from tuberous roots according to the manufacturer's instructions accompanying the SMART RACE cDNA Amplification Kit (Clontech, USA) and amplified with templates of cDNA using a degenerated primer and adaptor primer. The remaining 5′-sequences of the three cDNAs were obtained by 5′-RACE with total RNA of developing tuberous roots.

Reverse Transcriptase (RT)-PCR

Total root RNA samples were treated extensively with RNase-free Dnase I to remove any contaminating genomic DNA. The first-strand cDNA was synthesized using Pfu Turbo polymerase (Stratagene, USA) from 2 μg of total RNA in a 20 μL reaction volume, and 2 μL of the reaction mixture was subject to subsequent PCR in a 50 μL reaction volume. *IbMADS79* (5′-TGGACA-GGTACGAGAGATACTCC-3′ and 5′-GGTGTCCAGC-TGTTGCTCTAAAC-3′), *IbAGL20* (5′-CTGAGAACC-CATCAGTGGAAC-3′ and 5′-CTCTCCAGCCTGT-TGCTCTATCTG-3′), *IbAGL17* (5′-CG-CA-ACAACTAG-CATCAGATCCG-3′ and 5′-GGAAGCTGGAAATGTA-TGTGGC-3′), *IbMADS 3* (5′-AATT-GCAGCTAGTGG-AGAACGC-3′ and 5′-CTTGTGG-AGGACTGGTCGA-GTT-3′), *IbMADS 4* (5′-GCAAGG-AGGTTGCTGA-CAAGA-3′ and 5′-CAGCGATGTATC-GGAGCAATC-3′), *AGPase* (5′-CGGGGATGAAGTGG-TTTCAGGG-3′ and 5′-GGGACTTTTCCATCTGCCAGAAGCG-3′) and *Tublin* (5′-CAACTACCAGCCACCAACTGT-3′ and 5′-CAAGATCCTCACGAGCTTCAC-3′) were amplified using the indicated primers according to the following cycling conditions: for the RT-PCR runs were 25 cycles with each cycle at 94°C for 0.5 min, 62°C for 0.5 min, and 72°C for 1 min, with a final cycle at 72°C for 5 min to allow the completion of the polymerizations.

Sequence Analysis

Plasmids were purified from selected colonies and sequences of both strands of inserted DNA were determined in full length with ABI Prism TM 3100 genetic analyzer (Perkin-Elmer Applied Biosystems, USA). Sequences were analyzed using GENETYX (Software Development, Japan) software package. The neighbour-joining method was used to generate a dendrogram of MADS box proteins.

RESULTS AND DISCUSSION

Isolation of Sweetpotato MADS Box Genes

For the isolation of sweetpotato MADS box genes

related to the root development and tuberization, total RNA was isolated from developing tuberous root from plants 50 d after planting. This RNA was subjected to RT-PCR using degenerated MADS box primers, starting with asymmetric amplification on first strand cDNA (synthesized from the poly A tail) with the degenerated primer of MADS box region. Bands between 600-800 bp were subcloned, and found to contain almost exclusively an MADS box cDNA fragment. Three different cDNA fragments were identified with similarity to known MADS box genes of other plants. The remaining 5′-sequences of the three cDNAs were obtained by 5′-RACE with total RNA of developing tuberous roots. Sequence analysis revealed that the three MADS box genes were homologous to *TM3*, *AGL17* and *SQUA* MADS box genes that are involved in vegetative tissue development. These three MADS box genes resembled genes implicated in the vegetative tissue development and determination of phase transition from vegetative to reproductive stage identity of other species.

Sequence Characterization of *IbAGL17*, *IbAGL20* and *IbMADS79*

The *IbAGL17* (Genebank accession number DQ011557) cDNA has the open reading frame of 219 amino acids. *IbAGL17* showed highest sequence similarity with a member of the *AGL17* clade of MADS box genes (Fig. 1, 2A). Especially, *ANR1* is related to the development of lateral roots in response to nitrate availability within the soil, thus linking environmental conditions and vegetative development (Zhang and Forde, 1998; Zhang et al., 1999). However, little information is available on *AGL16*, *AGL17* and *AGL21*.

The *IbAGL20* (Genebank accession number DQ020264) full length cDNA is 1232 bp long. The coding region is 662 bp, encoding a 220-amino acid protein (Fig. 2B). The 3′-non-coding region is 252 bp and the 5′-non-coding region 318 bp. *IbAGL20* fits within the *TM3* clade (Fig. 1). The genes of *TM3* clade are involved in the control of flowering time and mainly belong to the autonomous pathway, as demonstrated by a down-regulation of this gene in mutants affected in both the photoperiodic and the autonomous pathways (Lee et al., 2000).

IbMADS79 (Genebank accession number DQ020263) cDNA is 1147 bp long, with open reading frame of 725 b long encoding a protein of 241 amino acids. 5′- and 3′-non-coding regions are 194 and 227 bp, respectively. This cDNA is most similar to

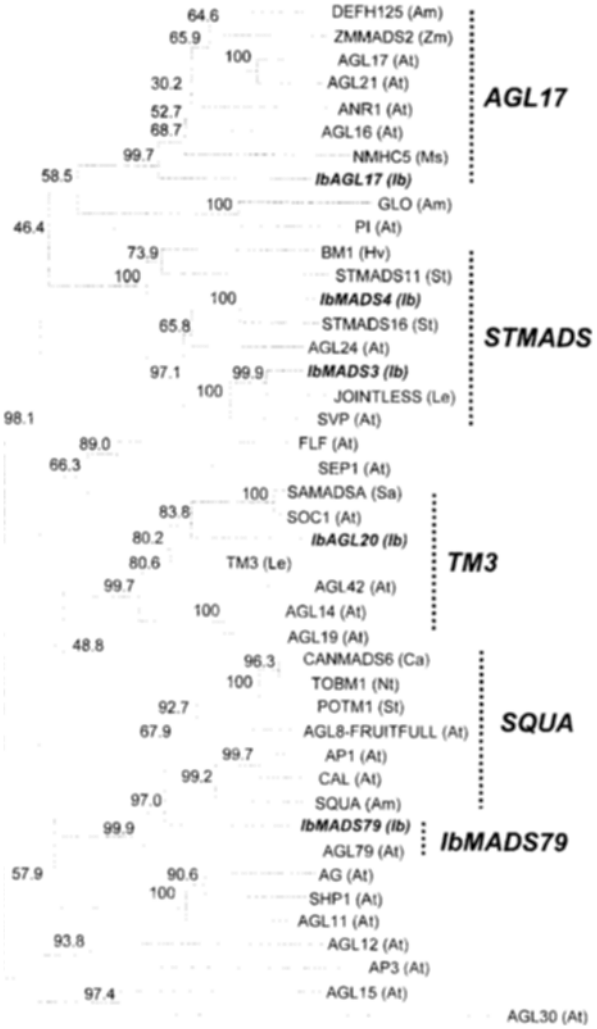

Figure 1. Phylogenetic tree of MADS box proteins from sweetpotato and different dicot species. The neighbour-joining method was used to generate a dendrogram of MADS box proteins. Bootstrap values expressed as percentage (over 1000 replicates) are shown at the corresponding nodes. Sources of the genes are indicated in parentheses follows. At, *Arabidopsis thaliana;* Am, *Antirrhinum majus;* Hv, *Hordeum vulgare;* Le, *Lycopersicon esculentum;* St, *Solanum tuberosum;* Ib, *Ipomoea batatas ;* Zm, *Zea mays;* Ms, *Medicago sativa;* Ca, *Capsicum annuum;* Nt, *Nicotiana tabacum;* Sa, *Sinapis alba.*

the *AP1* and *CAL* from *Arabidopsis*, and *SQUA* from *Antirrhinum*, and fits in the *SQUA* clade (Fig. 1, 2C). The genes of *SQUA* clade are related to the control of the flowering time and/or the specification of meristem identify (Huijser et al., 1992; Mandel et al., 1992; Kempin et al., 1995; Theißen et al., 1996; Gu et al., 1998; Parenicova et al., 2003) even though the *IbMADS79* was isolated from developing tuberous root.

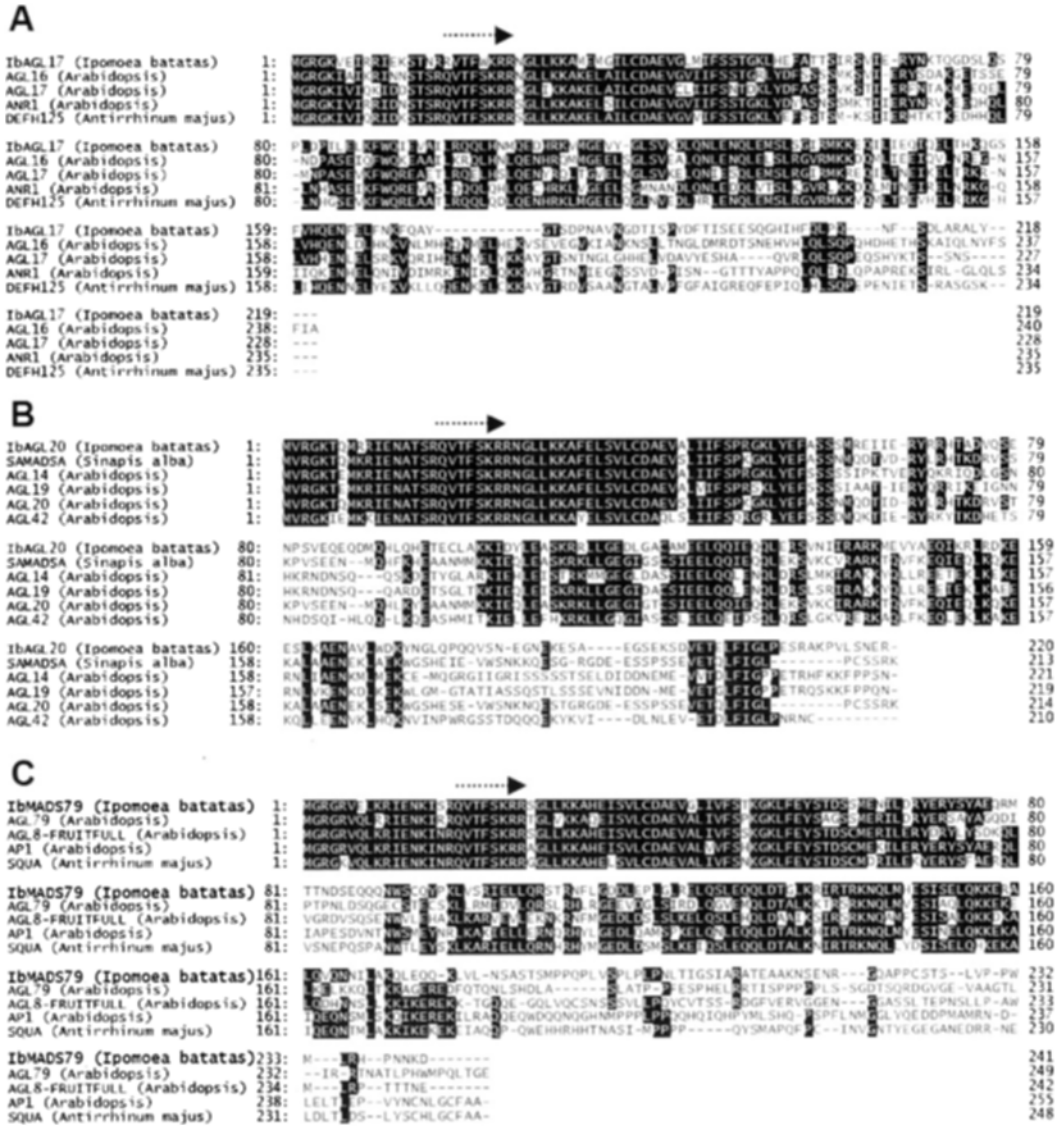

Figure 2. Deduced amino acid sequence of *IbAGL17* **(A)**, *IbAGL20* **(B)**, and *IbMADS79* **(C)**. Conserved residues are black boxed. Degenerated primer site is a dotted line.

RT-PCR Analysis of *IbAGL17, IbAGL20* and *IbMADS79*

Expression levels of *IbAGL17*, *IbAGL20* and *IbMADS79* mRNA were examined in the flower and different vegetative tissues on RT-PCR. In root tissues, white fibrous root, thick-pigmented root and tuberous root were also used for comparison. The gene-specific six pairs of primers were designed and are presented in Materials and Methods.

IbAGL17 mRNA was not detectable in the flower or leaf (Fig. 3). In the flower and leaf materials no signals were detected on the RT-PCR, even after extended PCR cycles (data not shown). Expression levels were highest in root tissues, especially tuberous root tissue, in agreement with the suggestion that *IbAGL17* is involved in the *AGL17* clade that genes of clade also expressed in root tissues.

Expression of *IbAGL20* was detected in all tissues, which is consistent with the suggestion that it is a *TM3* gene orthologue. *AGL20/SOC1* is expressed to some extent in most vegetative plant tissues during long day conditions, including the leaves, stems, apical meristems, inflorescence meristems, and to a small degree in roots, but it is also expressed in flowers (Lee et al., 2000).

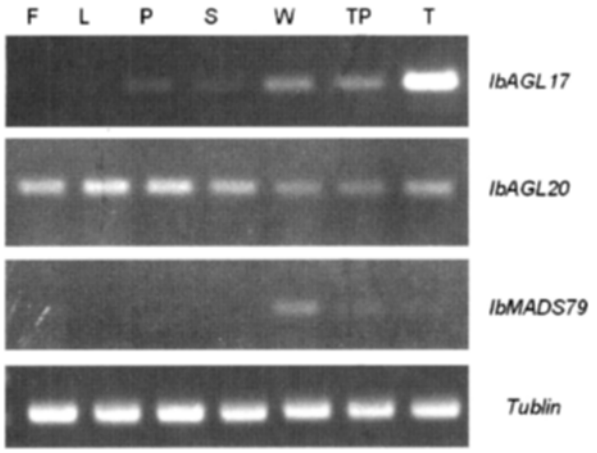

Figure 3. Expression analysis of *IbAGL17*, *IbAGL20*, and *IbMADS79*. RT-PCR analysis was performed on equal amounts of cDNA from flower (F), leaf (L), petiole (P), stem (S), white fibrous root (W), thick-pigmented root (TP) and tuberous root (T). *Tublin* was used as a control.

IbMADS79 expression in white fibrous root and in small degrees in thick-pigmented root and tuberous root does not agree with the expression pattern of *SQUA*, but sequence homologies are highest with *SQUA* and *SQUA*-homologues. Several *SQUA*-like genes have been identified: *AP1* (*Arabidopsis*) (Mandel et al., 1992), *CAL* (*Arabidopsis*) (Kempin et al., 1995), *AGL8* (*Arabidopsis*) (Gu et al., 1998), and *SQUA* (*Antirrhinum*) (Huijser et al., 1992). Cladistic analysis based on protein sequence comparison revealed that *IbMADS79* shows the highest similarity (52% overall and 94% homologue in MADS box region) to those genes of *SQUA* clade (Huijser et al., 1992). Within the same cluster are also found *AP1*, *TOBM1*, *POTM1*, *AGL8*, *CAL* and *SQUA*, these genes designated as *SQUA* clade. Recent comprehensive studies (Cseke and Podila, 2004) also grouped *AGL79* in the same cluster on the basis of particular amino acid deviations from the MADS-box consensus sequence. Following this criterion, only *IbMADS79* and *AGL79* would be considered within the same subfamily. Moreover, the expression patterns of *AP1* (whose transcript is present in flower, floral and vegetative meristem) (Mendel et al., 1992) and *AGL8* (mRNA mainly found in fruit and inflorescence meristem) (Gu et al., 1998) are different from those of *AGL79* and *IbMADS79*. All these data suggest that although they are most similar to *SQUA* and *SQUA* orthologues of other plants, *AGL79* and *IbMADS79* are not functional homologous of *SQUA* clade genes. Since *IbMADS79* was the first gene to be identified within this group of MADS-box gene, it has been suggested that this new subfamily be named the *IbMADS79* clade. Finally, the high similarity between *AGL79* and *IbMADS79* as well as the overlapping expression patterns make them candidates for involvement in the same developmental pathway.

Expression Pattern Analysis of Sweetpotato MADS Box Genes

The expression of the 5 kinds of sweetpotato MADS box gene at early stages of root development was analyzed by RT-PCR with gene-specific primers on RNA from whole root tissues from 15 to 60 d.

The expression of MADS box genes was compared with that of AGPase (ADP-glucose pyrophosphorylase) in the RT-PCR analysis of this study because AGPase is responsible for forming ADP-glucose, the substrate for starch synthesis (Müller-Röber et al., 1992). AGPase was also expressed only in developing tuberous root among the three kinds of roots in sweetpotato (white fibrous root, thick pigmented root, and tuberous root)

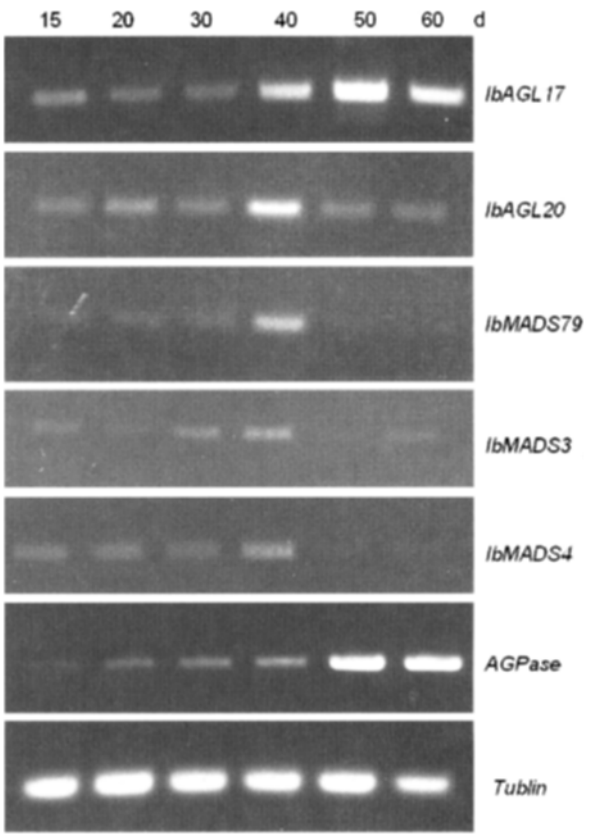

Figure 4. Developmental stage expression pattern of 5 MADS box genes and *AGPase* in total root tissues of sweetpotato. Total RNA was isolated from 15 to 60 d. *Tublin* was used as a control. d, days.

(Kim et al., 2002b). AGPase is a key enzyme for starch synthesis and marker for tuberization in sweetpotato. In the present study the AGPase gene expression pattern at different developmental stage suggests that AGPase is positively correlated to sink strength at normal growth condition (Li and Zhang, 2003).

In the previous study, two MADS box genes, *IbMADS3* (Genebank accession number AB054255) and *IbMADS4* (Genebank accession number AB054256), were isolated from the tuberous root of sweetpotato (Kim et al., 2002a). They showed different expression patterns in root tissues (Fig. 3), so that the expression pattern of *IbMADS3* and IbMADS4 were also demonstrated in different root development stage.

Fig. 4 shows that *IbMADS79* was weakly expressed from 15 d and from 50 d. *IbAGL20* mRNA was also observed from 15 to 60 d. Level of expression of this particular mRNA may be related to age and developmental stage. In root tissues at 15, 20 and 30 d, the amount of PCR product appeared to be generally lower than those of 40 d root tissues, but the signal was not detectable at 50 d. For both AGPase and *IbAGL17*, the strongest expression occurred in root tissues from 50 d. The main difference occurred with regard to expression; the RT-PCR analysis detected relatively active expression in the case of AGPase. The results indicated that *IbAGL17* demonstrated enhanced expression in tuberous root and were most active at the enlarging stage of the immature tuberous root at which rapid accumulation of starch and abnormal vascular cambium activity for tuberization is expected.

Sweetpotato roots stopped elongating at about 40 d after planting and started to thicken. The total dry weight of the root rapidly increased, reaching about five times of the initial value (Kim et al., 2002a) meaning of close relationship between starch accumulation and cell proliferation for tuber bulking during root development. *IbAGL17* transcript is mainly found in vascular bundles of petioles and developing tuberous roots (data not shown), and it is tempting to speculate the possible role of MADS box genes as a tuberization regulator in sweetpotato roots.

The present results clearly indicate that the activity of the MADS box genes in root tissues is not an unrelated coincidence to a particular developmental stage but is indeed correlated with sink strength during root development; therefore, we confirmed the suggested hypothesis that MADS box activity is associated with tuberization in sweetpotato. *IbAGL20*, *IbMADS79*, *IbMADS3* and *IbMADS4* may be expressed during the early stage of root development whereas *IbAGL17* dominates in the developing tubers. This result provides insight into the molecular pathways in storage-root of sweetpotato and other tuber crops.

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