Annals of Botany Page 1 of 11
doi:10.1093/aob/mcn067, available online at www.aob.oxfordjournals.org

OXFORD JOURNALS
OXFORD UNIVERSITY PRESS

IbMADS1 (*Ipomoea batatas MADS-box 1* gene) is Involved in Tuberous Root Initiation in Sweet Potato (*Ipomoea batatas*)

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Received: 18 January 2008 Returned for revision: 13 February 2008 Accepted: 31 March 2008

• *Background and Aims* The tuberization mechanism of sweet potato (*Ipomoea batatas*) has long been studied using various approaches. Morphological data have revealed that the tuberizing events result from the activation of the cambium, followed by cell proliferation. However, uncertainties still remain regarding the regulators participating in this signal-transduction pathway. An attempt was made to characterize the role of one MADS-box transcription factor, which was preferentially expressed in sweet potato roots at the early tuberization stage.
• *Methods* A differential expression level of *IbMADS1* (*Ipomoea batatas MADS-box 1*) was detected temporally and spatially in sweet potato tissues. *IbMADS1* responses to tuberization-related hormones were assessed. In order to identify the evolutionary significance, the expression pattern of *IbMADS1* was surveyed in two tuber-deficient *Ipomoea* relatives, *I. leucantha* and *I. trifida*, and compared with sweet potato. In functional analyses, potato (*Solanum tuberosum*) was employed as a heterologous model. The resulting tuber morphogenesis was examined anatomically in order to address the physiological function of *IbMADS1*, which should act similarly in sweet potato.
• *Key Results IbMADS1* was preferentially expressed as tuberous root development proceeded. Its expression was inducible by tuberization-related hormones, such as jasmonic acid and cytokinins. *In situ* hybridization data showed that *IbMADS1* transcripts were specifically distributed around immature meristematic cells within the stele and lateral root primordia. Inter-species examination indicated that *IbMADS1* expression was relatively active in sweet potato roots, but undetectable in tuber-deficient *Ipomoea* species. *IbMADS1*-transformed potatoes exhibited tuber morphogenesis in the fibrous roots. The partial swellings along fibrous roots were mainly due to anomalous proliferation and differentiation in the xylem.
• *Conclusions* Based on this study, it is proposed that *IbMADS1* is an important integrator at the initiation of tuberization. As a result, the initiation and development of tuberous roots seems to be well regulated by a network involving a MADS-box gene in which such hormones as jasmonic acid and cytokinins may act as trigger factors.

Key words: *Ipomoea batatas*, *Ipomoea* relatives, root, MADS-box, transcription factors, *IbMADS1*, tuberization, tuberous roots, sweet potato.

INTRODUCTION

Tubers are among the principal food crops in the world. Among these, sweet potato (*Ipomoea batatas*) is ranked seventh in importance and is characterized with fleshy, tuberous roots. Over recent years, considerable progress has been made in the isolation and characterization of genes associated with storage proteins, such as starch biosynthesis proteins (Wang *et al.*, 1999) and stress-related proteins (Wang *et al.*, 1995; Yeh *et al.*, 1997*a*, *b*; Yao *et al.*, 2001; Jih *et al.*, 2003). In order to understand how tuberous roots are developed, studies on morphogenesis have been carried out over a considerable period of time (Lowe and Wilson, 1974). Such work has clearly demonstrated that tuberous roots are originated from the activation of primary cambia, along with anomalous cambial activity in the secondary and vascular cambia. During the secondary-thickening growth, the procambial ring gradually aligns outwardly; meanwhile, parenchyma cells inside become highly proliferated and finally differentiate into starch-storage cells.

The initiation of organized development is a complex morphogenetic phenomenon, in which extrinsic and intrinsic factors play important roles (Aswath and Kim, 2005). Inputs from environmental cues, hormone signals and nutrient status are driving factors during organogenesis. In addition, the internal balance of plant hormones such as abscisic acid (ABA) and cytokinins is crucial to the phase transition and tuber development (Matsuo, 1983; Wang *et al.*, 2005). Such developmental programs mostly rely on the spatial and temporal regulation of transcription factors. Although many attempts have been made to identify genes involved in the formation of tuberous roots (You *et al.*, 2003; Tanaka *et al.*, 2004), there is still a paucity of molecular investigations on upstream regulators, such as MADS-box transcription factors.

The term 'MADS' is derived from *Minichromosomal maintenance 1* (*MCM1*), *Agamous* (*AG*), *Deficiens* (*DEF*) and *Serum response factor* (*SRF*) genes. These MADS-box transcription factors are named after a conserved N-terminal DNA binding domain (Garcia-Maroto *et al.*, 2003). Many of the plant MADS-box proteins are MIKC-type, including another conserved region that resembles the coiled-coil structure of keratin (K box), and two variable segments, the I region and C terminus (Pařenicová *et al.*, 2003). *In planta*, MADS-box transcription factors have been found extensively in floral organ

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differentiation, giving their function through either homo- or hetero-dimerization and transactivation (Kaufmann *et al.*, 2005). As a result, the 'ABCDE model' was proposed (Theißen, 2001; Theissen and Melzer, 2007). In addition to floral morphogenesis, recent findings have provided more evidence of MADS-box transcription factors in vegetative development (Rounsley *et al.*, 1995; Heard *et al.*, 1997; Carmona *et al.*, 1998; Montiel *et al.*, 2004). MADS-box genes of the *SQUA* (*SQUAMOSA*) subfamily appear to have a widespread expression pattern in both floral and vegetative organs. For example, *POTM1* (potato *MADS-box 1*) of potato (Kang and Hannapel, 1996) regulates the transitional phases from vegetative meristem to inflorescence meristem (Hart and Hannapel, 2002). In contrast, some subfamilies exhibit a strong tendency in vegetative expression; potato *STMADS11* and *STMADS16* (Carmona *et al.*, 1998; Garcia-Maroto *et al.*, 2000) in the *STMADS11* subfamily are one example in promoting vegetative growth. *AGL17* (*Agamous-like 17*) subfamily members, including *NMHC5*, *DEFH125*, *ANR1*, *AGL16*, *AGL17* and *AGL21* (Rounsley *et al.*, 1995; Heard *et al.*, 1997; Zhang and Forde, 1998; Alvarez-Buylla *et al.*, 2000; Burgeff *et al.*, 2002), are also specifically related to vegetative morphogenesis. As an example, *ANR1* is related to lateral root development in response to nitrate (Zhang and Forde, 1998; Alvarez-Buylla *et al.*, 2000). Thus far, MADS-box transcription factors have been verified as participating in diverged functional networks.

The purpose of the current study was to unravel the molecular mechanisms of tuberization in sweet potato. The cDNA-amplified fragment length polymorphism (cDNA-AFLP) technique was employed to isolate differentially expressed genes during tuberous root initiation. Among those genes, a transcript-derived fragment, *TDF26-1*, was cloned and identified as *Ipomoea batatas MADS-box 1* gene (*IbMADS1*) and subjected to further study. The gene expression pattern was examined and a transgenic study was carried out in order to address the physiological role of *IbMADS1* during the tuberization process in sweet potato.

MATERIALS AND METHODS

Plant material

Sweet potato (*Ipomoea batatas* L. Lam. 'Tainong 57' and 'Jewel', hexaploid), *Ipomoea leucantha* ('PI 540735', diploid), and *Ipomoea trifida* ('PI 561544', diploid) were cultivated in a growth chamber under conditions of a 8 h photoperiod, 30/25 °C day/ night. The sweet potato cultivar 'Tainong 57' was used in all studies except for the hormone treatments, in which 'Jewel' was used. Potato (*Solanum tuberosum* 'Désirée') for genetic transformation was propagated *in vitro* under conditions of a 16 h photoperiod, 25 ± 2 °C.

cDNA-AFLP analyses

Identification of differentially expressed transcripts was performed by the cDNA-AFLP technique as described by Bachem *et al.* (1996). Total RNA was isolated from leaves, roots and developing tuberous roots at different stages of growth: <2 g total plant fresh weight, 5–10 g, and approx. 50 g. The first and second strand cDNA synthesis was carried out with a SuperScript™ lambda system kit (Gibco BRL, Gaithersburg, MD, USA). After double digestion of cDNA by *Ase*I and *Taq*I, adaptors were ligated to restriction fragments for preamplification PCR. The resulting products were taken as templates in the selective amplification procedure. Combinatory PCR primer pairs, 5′-GACTGCGTACCTAATNN-3′ and 5′-ATGAGTCCTGACCGANN-3′, were designed according to preamplication primers with two base extentions (denoted as NN). Radioactive labelling of primers with [γ-^{33}P] ATP was performed as described by Vos *et al.* (1995). The amplified products were resolved on a 1 % sequencing gel and visualized by autoradiography at appropriate times. Differentially expressed transcript-derived fragments (TDFs) in early tuberous root samples were excised from the sequencing gel and rescued for sequencing. One particular TDF, *TDF26-1*, was further chosen for 5′-rapid amplification of the cDNA ends (5′-RACE) and 3′-RACE in order to reconstruct the full cDNA sequence by an overlapping method. The derived cDNA sequence of *IbMADS1* has been deposited into the NCBI Genbank data library under accession number AF396746 (2001), together with its corresponding amino acid sequence as AAK83920 (2001).

Cladistic analysis

A phylogenetic tree was drawn using MEGA version 3·1 with the neighbor-joining method and 1000 bootstrap replicates (Kumar *et al.*, 2004). MADS-box proteins were aligned using Clustal W version 1·83 (http://www.ebi.ac.uk/clustalw/index.html; Thompson *et al.*, 1994). The sequence data of MADS-box proteins is as follows: DEFH125 (CAA71739) from *Antirrhinum*; ANR1 (NP-179033), AGL14 (NP-192925), AGL16 (NP-191282), AGL17 (NP-179848), AGL19 (NP-194026), AGL21 (NP-195507), AGL24 (NP-194185), AP3 (NP-191002) and SVP (AAG24508) from *Arabidopsis*; POTM1-1 (Q42429), STMADS11 (AAB94006) and STMADS16 (AAB94005) from *Solanum tuberosum*; IbMADS3 (AAK27150), IbMADS4 (AAK27151), IbMADS10 (ABD66305), IbAGL17 (AAY29699), IbMADS79 (AAY84827) and IbAGL20 (AAY84828) from *I. batatas*.

Southern and Northern blot hybridization

Leaves were first ground into fine powder with liquid nitrogen, and plant genomic DNA was extracted by the acetyltrimethylammonium bromide (CTAB) -based method (Sambrook and Russell, 2001). Ten μg genomic DNA was separately digested with *Eco*RI and *Hind*III, resolved on a 0·8 % agarose gel under 50 eV for 2 h, and transferred to a Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech, USA) with transfer buffer (20× SSC).

The pine-tree method (Sambrook and Russell, 2001) was applied for total RNA extraction. Samples were collected

from various vegetative tissues, including leaves, stems, roots and various sizes of developing tuberous roots. The RNA sample was loaded at about 10 μg per lane, resolved on a 1 % agarose/formaldehyde gel under 50 eV for 1 h, and transferred to a Hybond-N^+ nylon membrane.

A specific 380-bp fragment, spanning the variable C domain and 3′ untranslated region (UTR) of *IbMADS1* (640–1020 bp), was amplified by PCR using the primer pair 5′-CAGGCATATGGCACAAGTGAC-3′ (forward) and 5′-GTTTATTTAACATCAAACACCAA-3′ (reverse) to exclude cross-hybridization with other MADS-box genes. The DNA probe was labelled with [α-^{32}P] dCTP and then was applied for hybridization. Hybridization with the reagent FastHyb (BioChain, USA) and probe-labelling with RediPrime™ II Random Prime DNA Labeling System (Amersham Pharmacia Biotech, USA) were performed according to the manufacturer's instructions. Following hybridization, filters were washed twice at low stringency (2× SSC, 0·1 % SDS) at room temperature and once at high stringency (0·1× SSC, 0·1 % SDS) at 60 °C. Images were captured using a Typhoon 9400 scanner (Amersham Pharmacia Biotech, USA) after autoradiography.

Semi-quantitative reverse-transcription PCR

Reverse-transcription PCR (RT-PCR) was performed with a one-step RNA PCR kit (TaKaRa, Japan). A fixed quantity of 1 μg total RNA was taken as the PCR template. After reverse transcription at 50 °C for 45 min, target transcripts were amplified with specific primers in a PCR amplification procedure. *18S rRNA* was applied as an internal control within each RNA sample with the primer pair 5′-GTGACGGGTGACGGAGAATTA-3′ (forward) and 5′-ACACTAAAGCGCCCGGTATTG-3′ (reverse). The derived products were resolved on a 1 % agarose gel for semi-quantitative analysis.

In situ hybridization

The procedure of *in situ* hybridization was performed as described by Kao *et al.* (2006). In brief, the root tissues of sweet potato 'Tainong 57' were fixed in 4 % paraformaldehyde, dehydrated serially and sectioned to 10 μm thick. Digoxigenin (DIG) -labelled probes were separately transcribed with T7 and SP6 polymerase from PCR-derived cDNA fragments. After dewaxing with xylene and washing with ethanol, slides were dried at 60 °C for 30 min before hybridization. Slides were then submerged in hybridization buffer (10 % Denhard's buffer, 20 % formamide, 2× SSC, 1 mg mL^{-1} salmon sperm DNA, 0·2–1 ng μL^{-1} DIG-labelled probe) and hybridized at 37 °C for at least 16 h. Colour detection was performed using BCIP/NBT according to the manufacturer's instructions (Boehringer Ingelheim, Germany).

Hormone treatment

Fibrous roots of sweet potato 'Jewel' were collected from *in vitro* plants and transferred to flasks containing MS liquid medium (Murashige and Skoog, 1962) supplemented with the hormones abscisic acid (ABA), 6-benzylaminopurine (BA) and jasmonic acid (JA) at different concentrations: 1, 10, 20, 50, 100 and 200 μM. MS liquid medium without hormone supplement was employed as the control. The short-term treatment was kept in darkness for 3 h and subjected to RNA blot analysis. The long-term treatment was kept in darkness for at least 6 weeks under the different hormone combinations. The cut ends of fibrous roots were exposed to media containing 1 and 10 μM of JA, while the other ends were submerged in media containing 1, 10, 20 or 50 μM of BA. Tuber-forming roots were collected for detection of gene expression by RT-PCR. In addition to *IbMADS1*, both tuber-specific expression genes of sweet potato were investigated as marker genes; one is *sporamin* (Yeh *et al.*, 1997*a*, *b*) and the other is *IbAGPase* (Wang *et al.*, 1999), which functions in the starch biosynthetic pathway. Two combinatory primer sets, 5′-CAACATGAAAGCC CTCGCA-3′ (forward)/5′-CTCAACACCGGTGCTCATTA GAT-3′ (reverse), and 5′-CGGGATGAAGTGGTTTCA GGG-3′ (forward)/5′-GGGACTTTTCCATCTGCCAGAA GCG-3′ (reverse) were applied to detect *sporamin* and *IbAGPase*, respectively.

Gene construction of 35S::IbMADS1

A full-length *IbMADS1* cDNA (1022 bp), encoding 218 amino acids, was amplified with the primer pair, 5′-GCTCTAGAGATTTCCATGTTGGTGT-3′ (forward) and 5′-CGGGATCCCAGTTTATTTAACATCA-3′ (reverse). The cDNA fragment was cloned in *Xba*I/*Bam*HI restriction sites of the pBI121 binary vector (Clontech, USA) driven by a CaMV 35S promoter.

Generation of Agrobacterium*-mediated transgenic potato lines*

Potato 'Désirée' was transformed with the *IbMADS1* gene. The vector pBI121 (control) and *35S::IbMADS1* plasmid were delivered to *Agrobacterium tumefaciens* strain EHA105 by electroporation. Shoot internodes from 1-month-old *in vitro* plantlets were taken as explants for transformation; the transformation process was performed as described by Wu *et al.* (2003). After selection on media containing 100 mg L^{-1} kanamycin, putative transgenic plants were assessed using genomic PCR and RT-PCR.

Histological studies

In order to clarify vascular developmental changes in transgenic potato roots, microscopic observations were performed. Tissue samples for anatomical observation were fixed in formaldehyde/acetic acid (FAA) solution (ethanol : water : formaldehyde : acetic acid = 50 : 35 : 10 : 5, v/v), dehydrated in a graduated ethanol series and embedded in paraffin wax. Cross-sections of 10 μm thickness were obtained, dewaxed with xylene, stained in Safranin O, and counter-stained in aniline blue. Images were acquired with a digital camera

(Nikon, Japan) and were processed and analysed using the image analysis software, Image J (Eliceiri and Rueden, 2005).

RESULTS

Isolation and molecular characterization of a MADS-box gene based on cDNA-AFLP

In order to identify upstream factors involved in regulating tuberization, the cDNA-AFLP technique was conducted to investigate temporal gene expression during sweet potato tuber formation. Several TDFs, expressed mainly in fibrous roots and early tuberous roots, were identified and retrieved from the gel. Among them, *TDF26-1*, 202 bp in size, was specifically amplified with the primer pair 5′-GACTGCGTACCTAATAC-3′ (forward) and 5′-ATGAGTCCTGACCGACC-3′ (reverse) in selective amplification. As the cDNA-AFLP profile showed (Fig. 1), the transcript had the highest expression level at the initial tuberization stage, but this gradually decreased as tuberous root development proceeded. After extension by RACE from both 5′- and 3′-ends, a full-length cDNA of 1022 bp was obtained by perfect overlapping. Being the first submission of a sweet potato MADS-box gene to Genbank, this was named as *Ipomoea batatas MADS-box 1* gene (*IbMADS1*; accession number AF396746).

The phylogenetic tree revealed that *IbMADS1* fell into the *AGL17* subfamily, as described by Becker and Theißen (2003; Fig. 2A); this subfamily was characterized for vegetative expression pattern (Theißen, 2001). Comparing IbMADS1 to other AGL17 subfamily members, the amino acid identity and similarity to the entire protein were as follows: 52·3 % and 69·6 % to DEFH125 from *Antirrhinum*; 52·0 % and 72·9 % to AGL16; 49·8 % and 69·2 % to AGL17; and 48·8 % and 67·5 % to AGL21 from *Arabidopsis*. Similar to these subfamily members, *IbMADS1* was a typical MIKC type II MADS-box gene. With 126-bp 5′-UTR and 239-bp 3′-UTR, the 657-bp ORF comprised the MADS-box, intervening region, K-box and variable C-terminus (Fig. 2B). A comparison was also made with other identified MADS-box proteins in sweet potato, such as IbAGL17, IbAGL20, IbMADS3, IbMADS4, IbMADS10 and IbMADS79 (Kim *et al.*, 2002, 2005*a*, *b*; Lalusin *et al.*, 2006; Fig. 2B). Among them, IbAGL17 showed the highest identity and similarity: 93·1 % and 94·5 %, respectively (Fig. 2B).

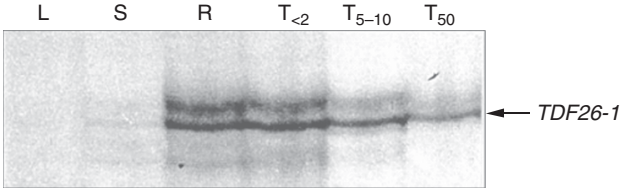


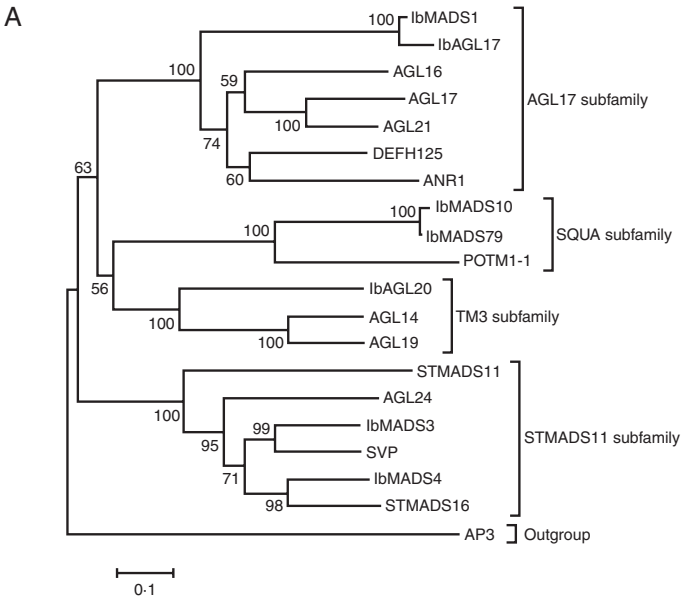
FIG. 1. Autoradiography of *TDF26-1* (arrowed) in a cDNA-AFLP profile. The cDNA-AFLP was performed in order to identify early tuber-specific genes. Total RNA was isolated from leaves (L), stems (S), fibrous roots (R) and developing tuberous roots (T), the latter ranging from less than 2 g ($T_{<2}$), 5–10 g (T_{5-10}) and approx. 50 g (T_{50}) in weight.

Hormone regulation of gene expression and tuberous root formation

It is well known that tuberization is controlled by several plant hormones (Wang *et al.*, 2005). Three kinds of tuberization-related hormones, ABA, JA and cytokinins, were tested for *IbMADS1* responsiveness. Fibrous roots were cultured in MS liquid media containing various concentrations of plant hormones for 3 h. ABA showed a relatively weak effect on *IbMADS1* induction (Fig. 3A), whereas the *IbMADS1* transcription level accumulated in accordance with increasing concentration of JA and BA, reaching a maximal expression level at 200 μM (Fig. 3A). Combinations of JA and BA at lower concentrations were applied in a long-term culture for *in vitro* tuberization. After 6 weeks of cultivation, several swellings with tuber-like appearance were observed from the fibrous roots under the hormone combination of 10 μM JA and 10 μM BA (Fig. 3B) as well as the combination of 10 μM JA and 20 μM BA (data not shown), whereas JA or BA alone at the concentrations tested (1, 10, 20, 50 μM) were not sufficient to initiate tuberous swellings on roots (data not shown). In accordance with tuberous root initiation, *IbMADS1* transcripts were detected copiously in the swollen root tissues (Fig. 3B). Up-regulation of two tuber marker genes, *sporamin* (Yeh *et al.*, 1997*a*, *b*) and *IbAGPase* (Wang *et al.*, 1999), was also detected (Fig. 3B). The former gene encodes the major storage protein, sporamin, in tuberous roots of sweet potato (Maeshima *et al.*, 1987; Yeh *et al.*, 1997*a*, *b*), whilst the latter encodes AGPase for ADP-glucose production in starch biosynthesis during tuber development. The result thus suggest a close correlation between *IbMADS1* expression and tuber morphogenesis.

Examination of IbMADS1 *expression patterns in* Ipomoea *species with regard to evolutionary relationships*

Two close relatives of sweet potato, *Ipomoea leucantha* (diploid) and *I. trifida* (diploid, tetraploid, hexaploid), have been considered as the putative progenitors of sweet potato without tuberous root formation (Saranya *et al.*, 2006). In order to investigate *IbMADS1* homologues among *Ipomoea* species, Southern and Northern hybridizations were carried out with an *IbMADS1*-specific probe. Since this probe contained neither the *Eco*RI nor *Hind*III restriction site, the Southern blot could reflect the theoretical number of *IbMADS1* homologues in the genome. In genomic analysis (Fig. 4A), hexaploid sweet potato exhibited multiple hybridization signals, while diploid *I. leucantha* and diploid *I. trifida* displayed a low copy number within the genome. As for *Arabidopsis* and potato, no hybridization signal was detected (data not shown). At the transcriptional level, *IbMADS1* transcripts were detectable in the fibrous roots of sweet potato, and showed highest accumulation at the stage of early tuber formation (Fig. 4B, $T_{<5}$). In contrast, the expression level of the *IbMADS1* homologue was too low to be detected in all tissues examined from *I. leucantha* and *I. trifida* (Fig. 4B). The results showed that the expression of *IbMADS1* was tuber organ-dependent. Moreover,



B

MADS-box

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IbMADS1     MGRGKVEIRRIEKSTNRRVTFWKRRNGLLKKAMEMGILCDAEVGLMIFSSTGKLHEFAT-  60 (aa)
IbAGL17     MGRGKVEIRRIEKSTNRRVTFWKRRNGLLKKAMEMGILCDAEVGLMIFSSTGKLHEFAT-
AGL16       MGRGKIAIKRINNSTSRQVTFSKRRNGLLKKAKELAILCDAEVGVIIFSSTGRLYDFSS-
AGL17       MGRGKIVIQKIDDSTSRQVTFSKRRKGLIKKAKELAILCDAEVCLIIFSNTDKLYDFAS-
IbAGL20     MVRGKTQMRRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVALIIFSPRGKLYEFAS-
IbMADS3     MAREKIKIKKIDNITARQVTFSKRRRGLFKKAEELSVLCDADVALIIFSSTGKLFDYAS-
IbMADS4     MAREKIKIKKIDNITARQVTFSKRRRGLFKKAEELAVLCDADVALIVFSATGKLFEFAS-
IbMADS10    MGRGRVELKRIENKISRQVTFSKRRSGPLKKAHEISVLCDAEVGLIVFSTKGKLFEYSTD
IbMADS79    MGRGRVELKRIENKISRQVTFSKRRSGLLKKAHEISVLCDAEVGLIVFSTKGKLFEYSTD
            * * :  :::*:.   *:*** *** * :*** *:.:****:* :::**  .:*.::::
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K-box

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IbMADS1     TSIRSVIERYNKTQGDSLQSPLDPTLE----LKFWQIEVAI-LRQQLHNMQEDHRKVMG-  120
IbAGL17     TSIRSVIERYNKTQGDGLQSPLDPTLE----LKFWQIKVAI-LRKQLHNMQEDHRKAMG-
AGL16       SSMKSVIERYSDAKGET-SSENDPASE----IQFWQKEAAI-LKRQLHNLQENHRQMMGE
AGL17       SSVKSTIERFNTAKMEE-QELMNPASE----VKFWQREAET-LRQELHSLQENYRQLTGV
IbAGL20     SSMREIIERYRRHTADV-QSE-NPSVEQEQDMQHLQHETEC-LAKKIDYLEASKRRLLGE
IbMADS3     SSMKGILERRNLHSKNL-EKMDQPSLE----LQLVENANHSRLSKEIADMTHRLRQMRGE
IbMADS4     SNMKDILGKYELHSSNL-DQATQPSRE----LQL-ENSLHVRLSKEVADKTRELRQMKGE
IbMADS10    SSMENILDRYERYSYAEQRMTTNVSEQ----QQNWPCQYPK-LVSRIELLQRSTRNFLGD
IbMADS79    SSMENILDRYERYSYAEQRMTTNDSEQ----QQNWSCQYPK-LVSRIELLQRSTRNFLGD
            :.:.  : :             : : :     :         *  .:      *.   *

IbMADS1     EVYGLSVKDLQNLENQLEMSLSGIRMKKEQILIEQIQELTHKQGSFVHQENFELFNK-FQ  180
IbAGL17     EVYGLSVKDLLNLENQLEMSMSSIIMKKEQLLIDQILELTHQQRSIVLQENFELFNK-FQ
AGL16       ELSGLSVEALQNLENQLELSLRGVRMKKDQMLIEEIQVLN-REGNLVHQENLDLHKK-VN
AGL17       ELNGLSVKELQNIESQLEMSLRGIRMKREQILTNEIKELT-RKRNLVHHENLELSRK-VQ
IbAGL20     DLGACAMEELQQIEQQLERSVNIIRARKMEVYAEQIKRLRDKEESL-KAENAVLWDK-YN
IbMADS3     DLQGMSIEELQQLERSLETGLSRVIEKKGEKIMKEINELQ-QKGMNLMEEKERLTQQVMA
IbMADS4     ELQGLSLEELQKLEKRLENGLTRVLETKGERVVTEIATLQ-RKGAELMKENKQLKEK-MA
IbMADS10    DLEPLGLRELQSLEQQLDTGLKRIRTRKNQLMHESISELQ-KKERALQVQNNILAKQ--V
IbMADS79    DLEPLGLRELQSLEQQLDTGLKRIRTRKNQLMHESISELQ-KKERALQVQNNILAKQ---
            ::   .:. * .:*  *:  :  :   : :    .*  *  ::       ::  *  :

IbMADS1     --------------------AYGTSDPNAVNGDTISPYDFTISEESQGHIHFQLPQNFSDL  240
IbAGL17     --------------------AYGTSDPNAVNGDTISPYDFTISEESQGHIHFQLPQNFSDL
AGL16       LMHQQN--MELHEKVSEVEGVKIANKNSLLTNGL-----DMRDTSNEHVHLQLSQPQHDH
AGL17       RIHQEN--VELYKK-----AYGTSNTNGLGHHEL----VDAVYESHAQVRLQLSQPEQSH
IbAGL20     GLQPQQ--VS---------NEGNEKESA-----------EGSEKSDVETELFIGLPESRA
IbMADS3     ISNGQR--VTAVINSDNMLNEEGLSSESI-TNVCNSTSPPQDYDDSSDTSLKLGLPY---
IbMADS4     RVNGEKFPVIADVEAAGLIPEEGQSSESITTNVCSCNSGPPPEDDCSDTSLKLGLPI---
IbMADS10    ELEQQK--LVLNSASTSMPSQPLVSPLPLPNLTIGSIARATEAAKNSENRGQALPCSTSL
IbMADS79    -LEQQK--LVLNSASTSMPPQPLVSPLPLPNLTIGSIARATEAAKNSENRGQAPPCSTSL
                                        .                 .   .

IbMADS1     ARAL----------Y
IbAGL17     ARAL----------Y
AGL16       ETHSKAIQLNYFSFIA
AGL17       YKTSSN---------S
IbAGL20     KPVLSNE--------R
IbMADS3     ----------------
IbMADS4     ---------------N
IbMADS10    VPPWMLRHPNN----K
IbMADS79    VPPWMLRHPNN----K
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FIG. 2. Phylogenetic analysis and sequence alignment of MADS-box proteins from dicot species. (A) Phylogenetic tree of plant MADS-box proteins with vegetative expression. One floral MADS-box transcription factor, AP3 (Bowman *et al.*, 1989), was chosen as the outgroup. MADS-box subfamilies are indicated on the right. Bootstrap values (%) over 1000 replicates are indicated at the nodes. (B) Alignment and sequence comparison between IbMADS1 and related MADS-box proteins in sweet potato and *Arabidopsis*. Numbers on the right indicate the protein length in amino acids (aa). MADS-box and K-box domains are indicated by lines above the alignments. Stars under residues indicate the identity among sequences; dots indicate progressively lower levels of conservation.

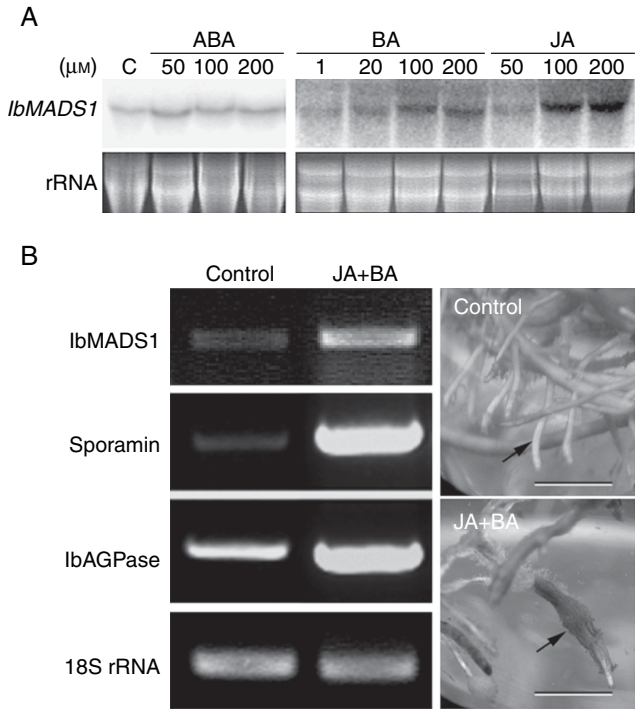

FIG. 3. Hormone effects on *IbMADS1* expression and tuberous root initiation in sweet potato. (A) Expression pattern of *IbMADS1* after hormone induction for 3 h. Northern blot analysis was performed on equal amounts of total RNA from hormone-treated fibrous roots of sweet potato 'Jewel'. Hybridization was performed with an *IbMADS1*-specific probe. Ethidium bromide (EtBr)-stained rRNA is shown as a loading control. The Northern analysis was performed twice with reproducible results. Abbreviations: C, no hormone; ABA, abscisic acid; BA, 6-benzylaminopurine; JA, jasmonic acid. (B) *In vitro* tuberous root initiation by combined treatment with 10 μM JA and 10 μM BA for 6 weeks. Relative expressions of *IbMADS1* and tuber marker genes in the regions indicated with arrows (images to right) were examined using RT-PCR (left). Sporamin, tuber storage protein in sweet potato (Yeh *et al.*, 1997*a*, *b*); IbAGPase, ADP-glucose pyrophosphorylase in sweet potato tuberous roots (Wang *et al.*, 1999). Constitutively expressed *18S rRNA* was used as an internal control. The arrows on the fibrous roots in the images on the right indicate swollen portions in the JA plus BA treatment. Scale bars = 5 mm.

IbMADS1 seemed to be specifically expressed in sweet potato among *Ipomoea* species. Subsequently, *in situ* hybridization was performed to localize the transcripts of *IbMADS1* in fibrous roots of sweet potato (Fig. 5); signals in blue-purple indicate the location of *IbMADS1* transcripts. In transverse sections, signals were mostly restricted to the stele, especially around the primary cambium (Fig. 5B). In a magnified view, *IbMADS1* was observed to be clearly localized in the emerging lateral root primordium (Fig. 5D) and immature meristematic cells such as the protoxylem and protopholem within the root stele (Fig. 5E). As lateral roots protruded, signals were similarly found in immature vascular cells (data not shown). The tissue-specific and cell-specific expression pattern strongly indicated that the functional role of *IbMADS1* was closely related to tuberization process.

Morphological examination of potato expressing IbMADS1

To further characterize *IbMADS1* function, we generated a gene construct harbouring full-length *IbMADS1* cDNA driven by a CaMV 35S promoter for plant transformation. Internodes from 6-week-old potato culture were infected by *Agrobacterium tumefaciens* EHA105 harbouring *35S::IbMADS1*/pBI121. Finally, three independent transgenic potato lines were chosen for phenotypic studies.

It is interesting to note that potato transgenic lines displayed a higher frequency of heavily swollen nodes, sessile microtubers and fused microtubers near basal nodes in *in-vitro* plantlets (data not shown); such morphological changes were similar to those mentioned by Thijn (1959). On the other hand, fibrous roots displayed unusual root tip swellings (data not shown) and partial bulking along the root axial in transgenic lines (Fig. 6B); the swollen size was approx. 50 % larger than the native roots from macroscopic observations (Fig. 6A, B). Anatomical data showed that the typical triarch pattern in the potato root stele (Fig. 6C) was altered into an anomalous poly-arch pattern in the swollen portion of transgenic potato roots (Fig. 6D). Measurements of the root width indicated that the stele enlarged from $84{\cdot}3 \pm 6{\cdot}4$ μm to $132{\cdot}5 \pm 16{\cdot}0$ μm, with the cortex region expanding from $234{\cdot}1 \pm 52{\cdot}8$ μm to $374{\cdot}5 \pm 80{\cdot}2$ μm. The expansion ratio was $1{\cdot}57 \pm 0{\cdot}005$ in the stele and $1{\cdot}60 \pm 0{\cdot}032$ in the cortex, which shows an equally proportioned increase in both stele and cortex (Fig. 7A). Cells in the stele with a differentiated secondary cell wall were counted for quantitative analyses. The multiplication of metaxylem cells from an average of $40{\cdot}3 \pm 3{\cdot}4$ to $82{\cdot}3 \pm 6{\cdot}3$ seemed to be the main cause for stele expansion (Fig. 7B). Meanwhile, the rest of the parenchyma cells within stele changed in number from $187{\cdot}7 \pm 16{\cdot}8$ to $204{\cdot}8 \pm 10{\cdot}6$.

DISCUSSION

A global gene analysis by cDNA-AFLP (Fig. 1) was carried out to screen the genes involved in the tuberization process of sweet potato. *IbMADS1* of the *AGL17* subfamily was selected for detailed characterization. In this study, we have provided molecular evidence to identify its physiological role in relation to tuberous root formation in sweet potato. The results have elucidated the possible function of *IbMADS1* in the tuberization process.

Molecular characterization of IbMADS1

Subfamily members usually share a high sequence similarity, similar expression patterns and similar functions (Theißen *et al.*, 1996). *IbMADS1* is evolutionarily grouped into the *AGL17* subfamily (Fig. 2A), which is a representative with a vegetative expression pattern (Theißen *et al.*, 1996; Becker and Theißen, 2003). It was evident that *IbMADS1* was preferentially expressed in early developing tuberous roots, but not in flowers or leaves (Fig. 4B). In fibrous roots, *IbMADS1* expression was mainly restricted to young vascular cells around the procambium, such as the primary xylem and primary phloem (Fig. 5B, E) and the primordia portion of emerging branch roots (Fig. 5D). This implies that active expression of *IbMADS1* might trigger a signal-transduction pathway of cell proliferation and cell growth, that would

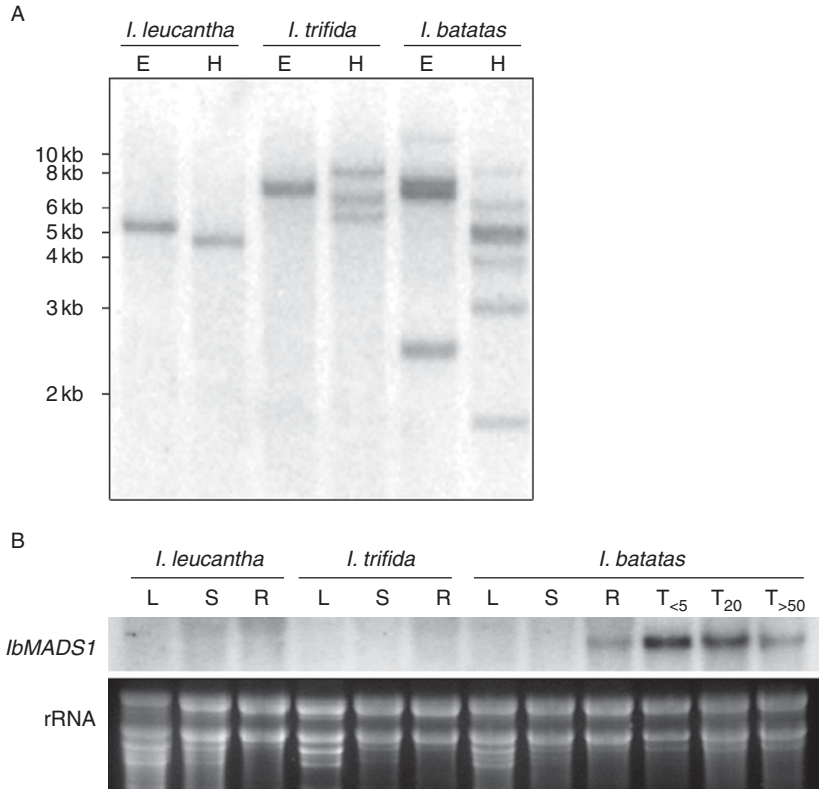

FIG. 4. Genomic analysis and expression patterns of *IbMADS1* among three *Ipomoea* species. (A) Southern hybridization of *IbMADS1* in *I. leucantha* (diploid), *I. trifida* (diploid) and *I. batatas* 'Tainong 57' (hexaploid). Restriction digested by *Eco*RI (E) and *Hind*III (H) −10 μg genomic DNA was loaded per lane. Size markers (kb) are indicated. A 380-bp *IbMADS1*-specific probe was applied in hybridization. (B) RNA transcripts of *IbMADS1* in vegetative tissues of sweet potato and its *Ipomoea* relatives. Total RNA was isolated from leaves (L), stems (S), fibrous roots (R) and developing tuberous roots ranging from <5 g ($T_{<5}$), 20 g (T_{20}), and >50 grams ($T_{>50}$) in weight; 10 μg total RNA was loaded per lane. The blot was hybridized with an *IbMADS1*-specific probe. Ethidium bromide (EtBr)-stained rRNA is shown as a loading control.

subsequently cause the outgrowth expansion of stele. The transduction signal might also activate starch biosynthetic genes to fulfill the storage function of the tissue. Our observations of *IbMADS1* expression in root vascular cells appear to coincide with previous reports on tuber morphogenesis (Lowe and Wilson, 1974).

Hormonal induction of IbMADS1 *expression and tuberous root initiation in sweet potato*

Plant hormones play a critical role during tuberous root initiation and development in sweet potato, for example cytokinins are essential throughout the tuberization process (Gan *et al.*, 2001). The effect of JA on root pigmentation is an early initializing signal for tuberous root formation (Gan *et al.*, 2001), while ABA is mainly associated with cell differentiation in the vascular cambium at later developmental stages (Wang *et al.*, 2005). The relatively strong induction by JA and relatively weak induction by ABA indicate the role of *IbMADS1* in mediating the initial tuberization stage. This finding matches the expression pattern of *IbMADS1* in sweet potato (Fig. 4B). To further determine the relationship, we successfully established an *in vitro* system to initiate tuberous roots by means of long-term hormone culture (Fig. 3B). The outcome was similar to what was reported by Nakatani (1994) in that *IbMADS1* was up-regulated in the bulking portion of the sweet potato roots (Fig. 3B). Two tuber marker genes, *sporamin* (Yeh *et al.*, 1997*a*, *b*) and *AGPase* (Wang *et al.*, 1999), were concomitantly expressed in the organs (Fig. 3B). The synergistic effect of cytokinins and JA on the onset of tuberization seems to be closely correlated with active expression of *IbMADS1*.

Evolutionary characterization in Ipomoea*-related species signifies the function of* IbMADS1

Among *Ipomoea* species, *I. trifida* has been recognized as the most likely progenitor of sweet potato (Saranya *et al.*, 2006). As a MADS-box gene member, *IbMADS1* was expressed mainly in early the tuberization stages of root tissues in sweet potato, whereas no transcript was detected in any examined tissue from *I. leucantha* and *I. trifida* (Fig. 4B). *IbMADS1* homologues may exist as silenced genes in the genomes of the two tuber-deficient *Ipomoea* relatives (Fig. 4A). A reasonable explanation of the novel character of extant sweet potato, i.e. the ability to form tuberous roots, is that it is because of cross-hybridization between *Ipomoea* species and self-polyploidization within the sweet potato genome – *IbMADS1* being activated in this process. From an

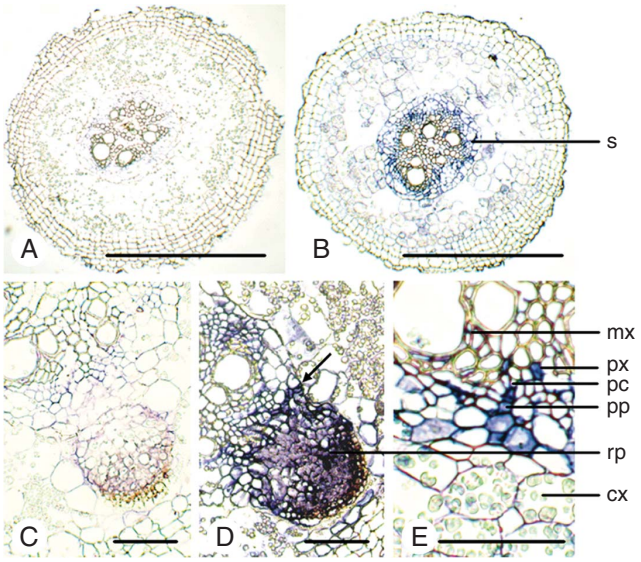

FIG. 5. Localization of *IbMADS1* RNA transcripts in fibrous roots of sweet potato. The digoxygenin-labelled probes of *IbMADS1* were employed in *in situ* hybridization: (A, C) sense riboprobe, (B, D, E) antisense riboprobe. All micrographs are pictured as transverse sections. (A) and (B) clearly demonstrate the centralized distribution of *IbMADS1* mRNA as blue-purple signals. Scale bars = 0·5 mm. (C), (D) and (E) show a magnification of *IbMADS1* RNA transcripts in the root primordium region, and immature vascular cells such as protoxylem and protopholem within the root stele. The arrow indicates the emergence of a lateral root. Scale bars = 50 μm. Abbreviations: cx, cortex; mx, metaxylem; px, protoxylem; pc, procambium; pp, protopholem; rp, root primordium; s, stele.

evolutionary perspective, we therefore propose that the active expression of *IbMADS1* is of great importance in conferring a physiological function related to tuberous root formation in sweet potato.

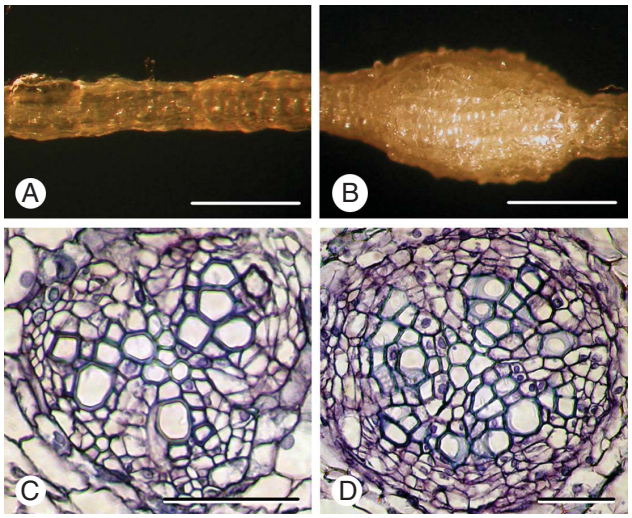

FIG. 6. The effect of *IbMADS1* expression on root morphological structure in potato. (A, B) Longitudinal views of potato roots, scale bars = 1 mm. (A) Potato transformed with vector only exhibits normal root morphology; (B) potato transformed with *35S::IbMADS1* displays partial swellings along roots. (C, D) Transverse sections in root steles, scale bars = 100 μm. (C) Dissection of roots from potato transformed with vector show a typical triarch stele. (D) Highly proliferated xylem cells cause an irregular arrangement, with a polyarch pattern being observed.

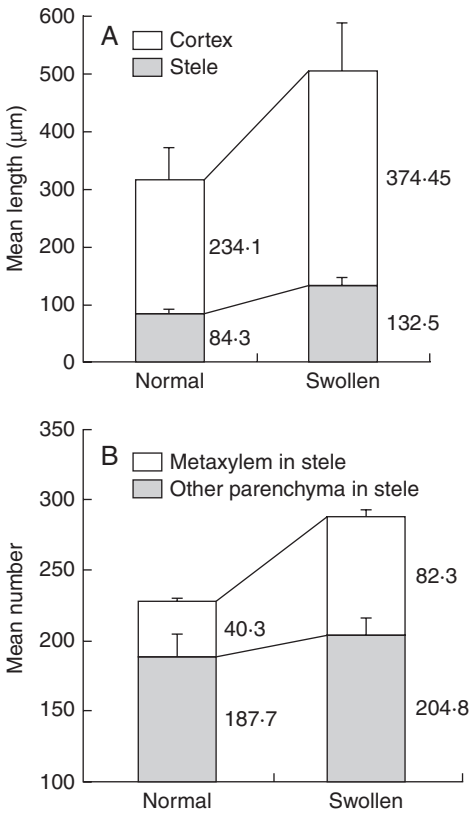

FIG. 7. Changes in root width and cell number of potato expressing *IbMADS1*. (A) The relative swelling in transgenic potato roots was compared between normal roots and swollen roots: the root sections were divided into cortex and stele, and their widths were measured (means ± s.e., $n = 12$). (B) The multiplication of cell numbers within the stele was compared between normal roots and swollen roots. Metaxylem cells with thickened cell walls and parenchyma cells within the stele, such as protoxylem, procambium and phloem, were counted (means ± s.e., $n = 12$).

IbMADS1 *expression in potato suggests a possible role in the tuberization-initiation process*

To date, many plant MADS-box transcription factors have been reported in controlling flowering processes and floral organ identity (Kaufmann *et al.*, 2005). Therefore, an attempt was made to express *IbMADS1* in *Arabidopsis*; however, we have not been able to find notable morphological alterations in the floral organ and flowering time (data not shown). As an alternative, we employed potato as the heterologous expression system in this study: potato already has a well-established plant transformation system, and the tuberization mechanisms in potato and sweet potato supposedly share general features and perhaps a general signal pathway. Although the origins of the tuber organs are different, potato tubers are analogous to sweet potato tuberous roots in many physiological aspects. For example, similar environmental and endogenous factors, such as high cytokinins and low nitrogen (Wilson and Lowe, 1973), will result in tuberization in both potato and sweet potato. According to the reports of Artschwager (1924) and Togari (1950), sweet potato tuberous roots were mostly derived from the structural precursors

of pentarch or hexarch steles and enlarged apical meristems. Interestingly, the development of tuberous roots from adventitious roots with poly-arch steles in sweet potato pheno-copied the morphological alterations observed in transgenic potatoes expressing *IbMADS1* (Fig. 6). Sweet potato experiences anomalous xylem proliferation from activated root procambium, especially at the beginning of tuberous root induction (Lowe and Wilson, 1974). In our histological study on transgenic potato, metaxylem doubling inside the stele of swollen roots (Fig. 7B) likewise demonstrated an enhanced activity in cell proliferation and xylem differentiation from young vascular cells. The resulting poly-arch steles in partially swollen roots (Fig. 6D) of transgenic potato occurred together with puffy root apical meristems at root tips in some cases (data not shown). In agreement with the above transgenic phenotypes, *IbMADS1* may trigger the tuberization potential of potato roots by incorporation into the signal-transduction pathway to activate downstream genes. In this way, alterations in stem-derived potato tubers would have a chance to take place, as mentioned above (data not shown). Since three potato MADS-box genes, *POTM1*, *STMADS11* and *STMADS16*, have been characterized with vegetative expression in roots and tubers (Kang and Hannapel, 1996; Carmona *et al.*, 1998; Garcia-Maroto *et al.*, 2000; Hart and Hannapel, 2002), we cannot exclude the interference of endogenous potato MADS-box genes; howevr, there are several arguments against such interference, as follows. First, *STMADS11* and *STMADS16* have no direct relationship with tuber morphogenesis. *STMADS11* is suggested to be a vegetative regulator involved in sprout initiation at the dormant meristem within the potato tuber (Carmona *et al.*, 1998). Ectopic expression of *STMADS16* confers vegetative features to flowers and also promotes vegetative growth in internodes and hence stem elongation (Garcia-Maroto *et al.*, 2000). Second, the axillary meristem has great plasticity to develop into shoots, stolons and tubers. *POTM1* mediates axillary meristem development, but not initiation (Rosin *et al.*, 2003). Suppression of *POTM1* results in an activated axillary meristem, increased cytokinin content and reduced tuber formation (Rosin *et al.*, 2003); however, morphological changes in either tuber or root have not been mentioned. Third, *POTM1* is proposed to favour the development of a dominant sink organ by regulating hormone balance (Rosin *et al.*, 2003). In this study (Fig. 3A), *IbMADS1* expression was regulated by cytokinins, instead of acting as an upstream regulator. It is conceivable that *IbMADS1* works differently from *POTM1* in potato, although further investigation is required. In other words, *IbMADS1* is anticipated to contribute to the proliferative potential in facilitating tuber organ initiation in both potato and sweet potato. In order to further demonstrate the function of *IbMADS1*, our future target is to characterize the tuber-forming potential of sweet potato in both over-expression and suppression lines. A transformation system for sweet potato is currently in progress based on our previous studies (Ashok Kumar *et al.*, 2007).

Co-ordinated regulation of MADS-box transcription factors in tuberous root formation

To date, several MADS-box genes have been isolated from sweet potato roots, such as *IbMADS3*, *IbMADS4*, *IbMADS10*, *IbAGL20* and *IbMADS79*, (Kim *et al.*, 2002, 2005*b*; Lalusin *et al.*, 2006). These MADS-box transcription factors may contribute to root development at different stages (Kim *et al.*, 2005*b*). The expression of *IbMADS3*, *IbMADS4* and *IbMADS79* has been detected mainly in fibrous roots before tuberous root formation (Kim *et al.*, 2002, 2005*b*), whilst *IbAGL20* is constitutively expressed in all tissues (Kim *et al.*, 2005*b*). *IbMADS10* has been reported in relation to anthocyanin accumulation in both flowers and pigmented root periderm and cortex tissue (Lalusin *et al.*, 2006). Our study has demonstrated that *IbMADS1* was predominantly expressed in developing tuberous roots (Fig. 4B). The diversified expression patterns of these *IbMADS* genes in sweet potato suggest that partial functional redundancy is an evolutionary product, rather than simply coincidence. Parallel expressions in sweet potato MADS-box genes have been observed as fibrous roots developed into tuberous roots (Kim *et al.*, 2005*b*). Root dry weight in sweet potato increased by a factor of five between 15 and 60 d after planting; at the stage of root thickening commencing at 40 d, *IbMADS3*, *IbMADS4*, *IbAGL20* and *IbMADS79* reached a maximal expression level (Kim *et al.*, 2005*b*). Transcripts of *IbMADS1* and *IbAGL17* were still accumulating up to 50 d and then gradually decreased (Kim *et al.*, 2005*b*). This phenomenon implies a promising mechanism linking vegetative *IbMADS* genes to the onset of tuberization. As proposed by Aswath and Kim (2005), co-ordinated expression of *IbMADS1* with other vascular developmental genes is a hallmark of the complexity of tuberous root induction. Our data provide support to the link between *IbMADS1* function and the tuberization process, providing an insight into the genetic network of *IbMADS1* and other *IbMADS* genes during the course of root development. After being triggered by some hormone signals, such as cytokinins and JA, these *IbMADS* genes may co-operate by interacting in dimers or tetramers in order to initiate a signal-transduction cascade that is involved in tuberous root organogenesis. In conclusion, this study provides basic knowledge and concepts on developmental windows that should help in the future improvement of the yields of sweet potato and other tuber crops.

ACKNOWLEDGMENTS

We thank the National Science Council, Taiwan, for the financial support to Dr Kai-Wun Yeh under the projects NSC-91–2317-B-002-005 and NSC-92–2311-B-002–024. We also thank the National Plant Genetic Resources Center, Taiwan, for the provision of seeds of *I. leucantha* and *I. trifida*. Great thanks also to Dr Chun-Neng Wang for assistance with the *in situ* hybridization technology and to Dr Hann-Chung Lo's lab for the help with microscopic manipulation.

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