

# Regulation of Expression of D3-type Cyclins and ADP-Glucose Pyrophosphorylase Genes by Sugar, Cytokinin and ABA in Sweet Potato (*Ipomoea batatas* Lam.)

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**Abstract** : The productivity of sweet potato is governed by both the rate of cell division and sink activity of its tuberous root. The aim of this study was to reveal the mechanisms that regulate cell division activity and sink activity during tuberous root formation. As an indicator of the cell division activity, we used the transcript level of two D3-type cyclins, which regulate cell cycle progression through the formation of the regulatory subunit of the cyclin-dependent kinase complex. As an indicator of photosynthetic product sink activity, we used the gene expression of ADP-glucose pyrophosphorylase (AGPase), one of the key enzymes of starch synthesis. During tuberous root formation, the expression of D3 cyclin genes increased to the maximal levels and then decreased. In contrast, the expression of the AGPase gene increased continuously. Sucrose enhanced the expression of D3 cyclin and AGPase genes, but a high concentration of sucrose repressed the expression of a D3 cyclin gene. In the presence of sucrose, cytokinin increased the expression of D3 cyclins, but abscisic acid (ABA) did not. However, cytokinin and ABA repressed the induction of AGPase gene expression by sucrose. These results suggested that sugars, cytokinin and ABA regulate the cell division activity and the sink activity in sweet potato.

**Key words** : Abscisic acid, ADP-glucose pyrophosphorylase, cytokinin, D3-type cyclin, gene expression, *Ipomoea batatas*, sugar, tuberous root formation.

Sweet potato (*Ipomoea batatas*) tuberous root is a commercially valuable organ that provides a high level of biomass and nutrients per hectare. Grafting experiments have suggested that the productivity of sweet potato is due to the sink strength of tuberous root, i.e. its capacity to deposit and store the products of photosynthesis (Hozyo et al., 1971; Harn, 1977). The sink strength of a tuberous root depends on: (i) the number of storage cells and hence the cell division activity in the tissue and (ii) the sink activity, that is, sugar transport and metabolic activity.

In the process of tuberous root formation, fibrous roots first develop the primary cambium between the protophloem and protoxylem (Kokubu, 1973; Wilson and Lowe, 1973). Subsequently, active cell division in the secondary meristem of the xylem results in a rapid increase of root diameter. The growing tuberous root is characterized by high sugar uptake and metabolic activity and as a result, has a high sink activity. The activity of ADP-glucose pyrophosphorylase (AGPase; EC 2. 7. 7. 27), which catalyzes the conversion of glucose-1-phosphate and ATP to ADP-glucose and pyrophosphate, is enhanced in the process of tuberous root formation and correlates positively to starch content (Nakatani and Komeichi, 1992).

The expression of a number of plant genes is

regulated by changes in sugar status via multiple signal transduction pathways (Smeeckens, 2000; Rolland et al., 2002). In general, the expression of genes involved in sink activities, such as genes coding for storage proteins and enzymes involved in starch synthesis, are upregulated by elevated sugar availability. Many of these genes are also regulated by other signaling molecules, such as phytohormones (Gibson, 2004). Levels of sucrose, cytokinins and abscisic acid (ABA) increase in the process of tuberous root formation of sweet potato (Matsuo et al., 1983; Nakatani and Komeichi, 1991; Saitou et al., 1997). Sucrose has been found to induce the expression of AGPase as well as sporamins, which represent almost 80% of the total soluble protein in tuberous root of sweet potato (Hattori et al., 1990, 1991; Bae and Liu, 1997; Harn et al., 2000). Analysis of the promoter region of the gene for sweet potato type-A sporamin identified a 210 bp sugar-inducible 'minimal' promoter (Morikami et al., 2005). ABA also induced the expression of the sweet potato sporamin gene (Ohto et al., 1992). These results suggest that sucrose and ABA play an important role in controlling the sink activity in the tuberous root of sweet potato. The regulation of cell division activity in sweet potato and coordination of the sink activity with the cell division in the process of tuberous root

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**Abbreviations** : AGPase, ADP-glucose pyrophosphorylase; BA, 6-benzyladenine; CDK, cyclin-dependent kinase; CycD, cyclin D; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction.

formation remain poorly defined.

Cyclins and cyclin-dependent kinases (CDKs) belong to the core cell cycle control machinery (Inzé, 2005). In higher eukaryotes, the association of D-type cyclins and a kind of CDK, CDKA, produces an active protein kinase that phosphorylates the retinoblastoma (Rb) protein at the G1/S transition. This phosphorylation results in the inactivation of Rb and the subsequent release of E2F transcription factor, which is responsible for the transcription of the S-phase genes (Nakagami et al., 2002). Overexpression of *Arabidopsis thaliana* *CycD3;1* or *Nicotiana tabacum* *CycD3;3* stimulates cells to exit the G1 phase, suggesting that D3-type cyclins act as rate-limiting regulators in the G1/S transition (Nakagami et al., 2002; Dewitte et al., 2003). *Arath;CycD3;1* is induced by sugars (Riou-Khamlichi et al., 2000), cytokinin (Riou-Khamlichi et al., 1999) and, to a lesser extent, by brassinosteroid (Hu et al., 2000) and other plant hormones, including auxin and gibberellin (Oakenfull et al., 2002). The expression of snapdragon D3 cyclin genes is enhanced by cytokinin and auxin, while that is diminished by brassinosteroid and ABA (Gaudin et al., 2000). *Arath;CycD3;1* protein and kinase activity parallel the abundance of its mRNA (Healy et al., 2001). These observations suggest that D3 cyclins modulate cell cycle progression in response to extracellular signals.

In this study, we isolated two cDNAs encoding D3 cyclins and investigated their expression in the process of tuberous root formation. To understand the molecular regulation of the cell division activity and the sink activity during tuberous root development, we examined the effects of sucrose, cytokinin and ABA on the expression of genes coding for two D3 cyclins and the AGPase large subunit.

## Materials and Methods

### 1. Plant material

Sweet potato (*Ipomoea batatas* Lam. cv. Koganesengan) was grown in a growth chamber under a 12-hr photoperiod with a photon flux density of 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at a relative humidity of 70%, at 25°C as described previously (Saitou et al., 1997). Apical cuttings with 4 unfolded leaves were taken from the stock plants, and all leaves were excised at the petiole-stem junction, except for the second and third leaves. The lower node of a stem cutting was inserted into vermiculite in a 4.5 L pot. Any outgrowths from the axillary buds were excised to maintain a single (unbranched) stem. After 4 wk, leaves, petioles, stems, fibrous roots (<2 mm in diameter), thick roots (2–5 mm in diameter), tuberous roots (>5 mm in diameter) and lateral roots emerging from tuberous roots were harvested separately. The samples were immediately frozen in liquid nitrogen and stored at –80°C.

### 2. Treatment of leaf-petiole cuttings

Petioles with the intact leaf attached, were cut from plants grown in the growth chamber. These leaf-petiole cuttings were dipped at their cut ends in distilled water at 25°C in darkness for 24 hr, and treated with distilled water, sugar, 6-benzyladenine (BA), or ABA solution for another 24 hr. Then, petiole portions of the cuttings were frozen in liquid nitrogen and stored at –80°C.

### 3. RNA isolation

Total RNA was isolated by the method described by Van Slogteren et al. (1983) with minor modification. Frozen tissue was powdered under liquid nitrogen and added to 10 volumes of 100 mM Tris-HCl, pH 8.0, 100 mM LiCl, 10 mM ethylenediaminetetraacetic acid and 1% (w/v) sodium lauryl sulfate made up as a 1:1 ratio with phenol and preheated to 80°C. The tissue extract was vortexed, mixed with 5 volumes of chloroform-iso-amyl alcohol (24 : 1), and vortexed again. After centrifugation at 10,000  $\times g$  for 15 min at 4°C, the supernatant was recovered and the RNA was precipitated by standing for 1 hr in LiCl at a final concentration of 2 M at –80°C. RNA was collected by centrifugation at 10,000  $\times g$  for 15 min at 4°C. The pellet was dried and resuspended in water, extracted once more with chloroform-iso-amyl alcohol, ethanol precipitated, and then resuspended in water. To remove any DNA contamination, we treated the total RNA with a Message Clean™ kit (Gen Hunter, Nashville, TN). The absorbance at 260 nm was used to quantify RNA.

### 4. Determination of full-length cDNA sequences

Total RNA was extracted from the thick roots of sweet potato and poly(A<sup>+</sup>) RNA was prepared from the total RNA by using an mRNA purification kit (GE Healthcare Bio-Science corp, Piscataway, NJ). Two partial cDNA fragments encoding D3-type cyclin were obtained by the 5' rapid amplification of cDNA ends (5'-RACE) method (Marathon™ cDNA Amplification Kit; Clontech, Palo Alto, CA). The following primers were used: as a reverse primer, 5'-GSACVVTS-GGTTTCTTCSACTTAGCAGC-3'; and as an adaptor primer, 5'-CCATCCTAATACGACTCACTATAGGGC-3'. The isolated cDNAs were termed *Ipoba;CycD3;1* and *Ipoba;CycD3;2*, respectively. The 3' regions of full-length cDNAs were obtained by the 3' rapid amplification of cDNA ends (3'-RACE) method according to the protocol of the TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (Takara Bio Inc., Otsu, Japan). The following primers were used: as a forward primer for *Ipoba;CycD3;1*, 5'-CTCAACTTCCTGCTCTCTGCTCTCTTCC-3'; as a forward primer for *Ipoba;CycD3;2*, 5'-CTTTCTCCCCTTGACTCTTCCACACCCC-3'; and as a 3' site adaptor primer, 5'-GTTTCCCAGTCACGAC-3'. The amplified cDNA fragments obtained from 5'- and 3'-RACE were cloned into pGEM-T Easy vectors (Promega, Madison,

Ipoba;CycD3;1	1	MFPLTHSQKD	QAFESRNPIF	SPDALFCEEE	RFEE--DVGG	WSRFQTDYYA
Ipoba;CycD3;2	1	M-----	---DARSPLT	SFDALYCEEE	QWGEVLDSEG	SGS-EVSEIP
Ipoba;CycD3;1	49	EISVLKPLL	TFLESDFWE	DDELLALLSK	EKDIGCVLDL	QSLMLARNEA
Ipoba;CycD3;2	38	RKEDSDSPVL	FLLDQDLCWE	DEELLSLFAK	ETDTH-LSFE	ASLSIPRTOA
Ipoba;CycD3;1	99	VDWMLKVIIR	YGFNALTAVL	AVNYFDRFIS	GVCFQRDKPW	MSQLAAVACV
Ipoba;CycD3;2	87	VRWILKVNNAH	YGFSPILTPTL	AVNYLDRFIS	GLQYQEDKPW	MIQLAAVACL
Ipoba;CycD3;1	149	SIAAKVEEIQ	VPLLLDLQVA	ETKFLFEAKT	IORMELLVLS	TLQWRMNLVT
Ipoba;CycD3;2	137	SLAAKVEETH	VPLLLDFQVE	DAEFVFDAKT	IQKMELLVLS	TLKWRMNPVT
Ipoba;CycD3;1	199	EISFIDHIIR	RFKLMTNLHW	EFLGLCERLI	LSVIADSRLL	QYLPSVVATA
Ipoba;CycD3;2	187	ELSFLDHITR	RLGLKNHLHW	EFFTKCESLI	LSFMPDSRFV	RFLPSVLATA
Ipoba;CycD3;1	249	IMFTVIKEIE	PCNAMKYKNE	LVCLLQISKE	KVDECYNLI	ELTGGKRNKQ
Ipoba;CycD3;2	237	TMLHVIHQLE	BSNAVYQNO	LLGLVKISKE	KVNDCYELIK	ELSTASANKR
Ipoba;CycD3;1	299	HCQHPKRKYE	PEPGSPNGVI	DAYFT-SDSS	NDSWAVCFSE	H-----KR
Ipoba;CycD3;2	287	SYSNLLCLLH	-DPNSESVAVI	DAVLSGCESS	NDSWGLETPE	PEQRRPLYKR
Ipoba;CycD3;1	342	TRI-----	-----	-----H		
Ipoba;CycD3;2	336	PRVEEQQMRV	PPLMTRVFVD	AIASPR		

Fig. 1. Multiple alignment of amino acid sequences of Ipoba;CycD3;1 and Ipoba;CycD3;2 of sweet potato. Dashes (-) indicate gaps introduced to maximize alignment and gray areas indicate identical amino acid residues. Stars show the residues proven to be essential for cyclin activity. The cyclin box is boxed, the retinoblastoma interaction motif is double underlined, and the putative PEST-destruction sequence is underlined.

WI) by the TA cloning method according to the manufacturer's protocol. We sequenced these cDNA clones by the dideoxy chain-termination method using an Applied Biosystems 373 Automated Sequencer (Applied Biosystems, Foster, CA). DNASIS-Mac software (ver. 3.0, Hitachi Software Engineering Co., Yokohama, Japan) was used for general sequence analysis.

##### 5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

First-strand cDNA was synthesized from 0.9  $\mu$ g of total RNA extracted from various sweet potato tissues as described above, using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (Takara Bio Inc.) with random hexamer primer, according to the manufacturer's instructions. Semi-quantitative RT-PCR was performed with 1/5 of the first-strand reaction mix, with the following gene-specific primers: 5'-GTCAACTTC-CTGCTCTCTGCTCTCTTCC-3' as a forward primer and 5'-TCTCGTTTCCAAACTGACGATTATGCTG-3' as a reverse primer for *Ipoba;CycD3;1*; 5'-CTTTCTCCCCT-TGACTCTTCCACACCCC-3' as a forward primer and 5'-TCCGGTCCGAGGTTTCTTGAAGATTCC-3' as a reverse primer for *Ipoba;CycD3;2*; 5'-GTCGTTCA-CACACTCCAAAGTCCGCAG-3' as a forward primer and 5'-TCCTCGGAGGTGGTGGTGGGACCAGACT-3' as a reverse primer for *iAGPLI-1* (accession number AF068260). As a loading control, the 18S rRNA gene

was amplified using primers, 5'-GCTTGTCTCAAA-GATTAAGCCATGCATG-3' as a forward primer and 5'-TCAGGCTCCCTCTCCGGAATCGAA-3' as a reverse primer. The PCR conditions used were: 94°C for 30 s; 59°C for 30 s; 72°C for 1 min 30 s. The reaction was repeated for 12–37 cycles to obtain an appropriate amount of DNA. The cycle numbers were determined to avoid the saturation of DNA amplification. PCR products were separated on a 1.5% agarose gel, followed by 1 hr staining with ethidium bromide. The image of the stained gel was recorded with a gel image analyzer (Molecular Imager FX; Bio-Rad, Ivry, France). The image density of each stained PCR product was analyzed using the software provided with the analyzer (Quantity One software; Bio-Rad). To confirm gene-specific amplification, we cloned and fully sequenced the amplified products.

## Results

### 1. Sequence analysis of sweet potato D3-type cyclins

Two full-length cDNAs encoding sweet potato D3-type cyclin were isolated from thick roots by RACE-PCR and were named *Ipoba;CycD3;1* (accession number AB478416) and *Ipoba;CycD3;2* (accession number AB478417) according to the conventions of Renaudin et al. (1996). *Ipoba;CycD3;1* and *Ipoba;CycD3;2* were 1201 bp and 1604 bp in length and contained open reading frames encoding putative proteins of 345 and

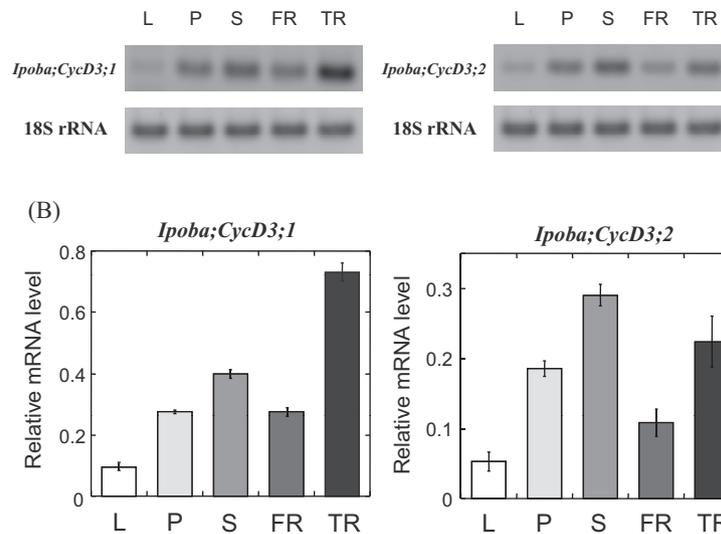


Fig. 2. Expression of the genes for *Ipoba;CycD3;1* and *Ipoba;CycD3;2* in different organs of sweet potato. Total RNA was isolated from leaf blades (L), petioles (P), stems (S), fibrous roots (FR), and tuberous roots (TR). (A) The mRNA levels were analyzed by RT-PCR in the exponential range of amplification. The PCR products were separated by agarose gel electrophoresis and photographed after ethidium bromide staining. (B) The relative mRNA levels of *Ipoba;CycD3;1* and *Ipoba;CycD3;2* were quantified from the images of gel electrophoresis, and normalized to that of 18S rRNA. Each value represents the mean  $\pm$  SE of results from three experiments.

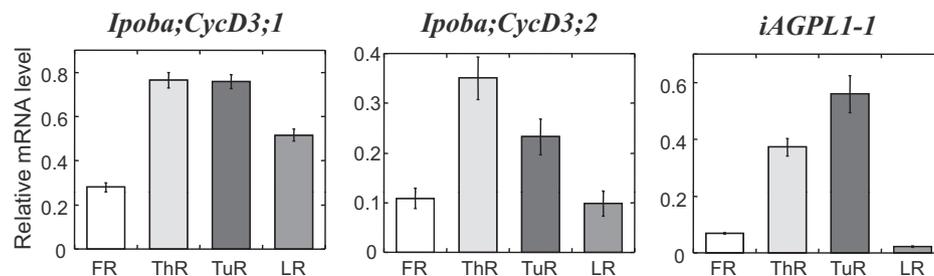


Fig. 3. Expression of genes for *Ipoba;CycD3;1*, *Ipoba;CycD3;2* and *iAGPLI-1* in different roots of sweet potato. Total RNA was isolated from fibrous roots (FR, <2 mm in maximum diameter), thick roots (ThR, 2–5 mm in maximum diameter), tuberous roots (TuR, >5 mm in maximum diameter) and lateral roots emerging from tuberous roots (LR). The mRNA levels of *Ipoba;CycD3;1*, *Ipoba;CycD3;2* and *iAGPLI-1* were normalized relative to 18S rRNA levels. Each value represents the mean  $\pm$  SE of results from three experiments.

361 amino acids, respectively. The deduced amino acid sequences of both *Ipoba;CycD3;1* and *Ipoba;CycD3;2* had the conserved cyclin box region of approximately 100 amino acids including the five key residues (R, D, L, K and E) for cyclin-CDK catalytic activity (Fig. 1). The retinoblastoma protein-binding motif LxCxE was present near the N-terminals of the two cyclins. Using the PESTFIND software (Rogers et al., 1986), we identified one potential PEST sequence, which specified rapid protein turnover (Murray et al., 1998), between positions 297 and 328 of *Ipoba;CycD3;1* with a PEST score of +6.88. No possible PEST sequence was detected in

*Ipoba;CycD3;2*.

## 2. Expression of D3 cyclin genes in various organs of sweet potato

The expression of the *Ipoba;CycD3;1* and *Ipoba;CycD3;2* genes was determined by semi-quantitative RT-PCR analysis using gene-specific primers. The relative abundance of transcripts in different organs varied with the D3 cyclin gene, although both genes were expressed in all organs tested: leaf blades, petioles, stems, fibrous roots and tuberous roots (Fig. 2). The highest level of *Ipoba;CycD3;1* transcript was detected in tuberous

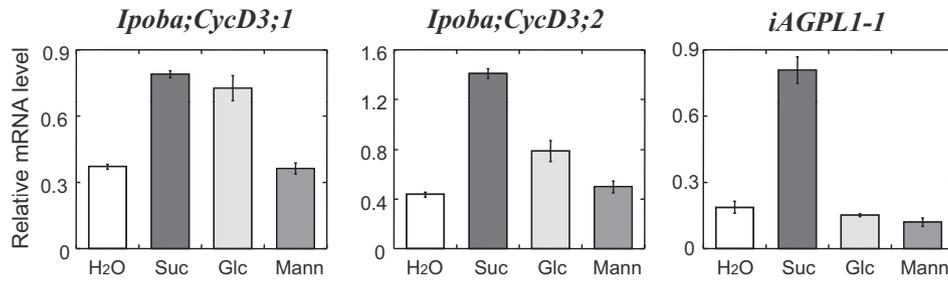


Fig. 4. Steady-state levels of mRNA for *Ipoba;CycD3;1*, *Ipoba;CycD3;2* and *iAGPLI-1* in leaf-petiole cuttings of sweet potato after treatment with sucrose, glucose or mannitol. Total RNA was isolated from the petiole portions of leaf-petiole cuttings that had been treated with 150 mM sucrose (Suc), 150 mM glucose (Glc), 150 mM mannitol (Mann) or water (H<sub>2</sub>O) for a day. The mRNA levels of *Ipoba;CycD3;1*, *Ipoba;CycD3;2* and *iAGPLI-1* were normalized relative to 18S rRNA levels. Each value represents the mean  $\pm$  SE of results from three experiments.

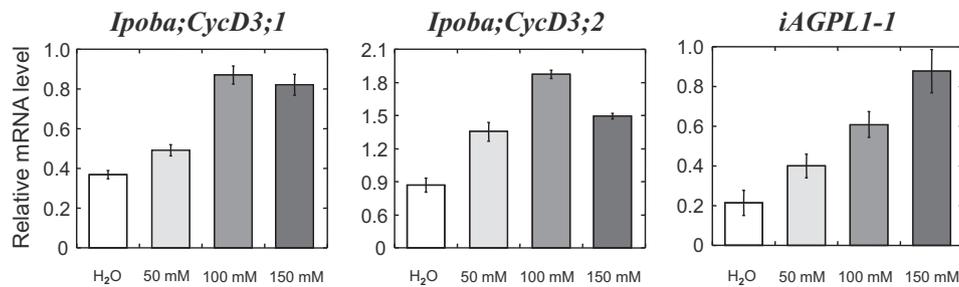


Fig. 5. Effects of various sucrose concentrations on expression of *Ipoba;CycD3;1*, *Ipoba;CycD3;2* and *iAGPLI-1* in leaf-petiole cuttings of sweet potato. Total RNA was isolated from the petiole portions of leaf-petiole cuttings that had been treated with 50, 100, 150 mM sucrose or water (H<sub>2</sub>O) for a day. The mRNA levels of *Ipoba;CycD3;1*, *Ipoba;CycD3;2* and *iAGPLI-1* were normalized relative to 18S rRNA levels. Each value represents the mean  $\pm$  SE of results from three experiments.

roots whereas *Ipoba;CycD3;2* was strongly expressed in stems and tuberous roots. Both *Ipoba;CycD3;1* and *Ipoba;CycD3;2* were expressed in leaf blades at comparatively low levels.

### 3. Expression of D3 cyclin and AGPase genes in the process of tuberous roots formation

Since the expression levels of *Ipoba;CycD3;1* and *Ipoba;CycD3;2* were high in tuberous roots, we further investigated the expression of these genes in the process of tuberous root formation. Roots were classified into four categories according to a previous anatomical study (Wilson and Lowe, 1973): fibrous roots (<2 mm in maximum diameter), thick roots (2–5 mm in maximum diameter), tuberous roots (>5 mm in maximum diameter) and lateral roots emerging from tuberous root. *Ipoba;CycD3;1* was expressed in thick roots and tuberous roots at the highest level (Fig. 3). The *Ipoba;CycD3;2* gene expression reached a maximum level in thick roots and was intermediate in tuberous roots.

AGPase catalyzes one of the main regulatory steps of starch biosynthesis in plants (Preiss, 1984). The transcript level of AGPase large subunit *iAGPLI-1*

(AF068260; Harn et al., 2000) was used as an indicator of the sink activity. The expression of *iAGPLI-1* increased in the process of tuberous root formation (Fig. 3) and its expression was very low in the lateral roots, which emerge from tuberous roots.

### 4. Effects of sugars on the expression of D3 cyclin and AGPase genes

The effects of various sugars on the expression of D3 cyclins and AGPase genes were examined by treating leaf-petiole cuttings separately with 150 mM sucrose, 150 mM glucose and 150 mM mannitol for 24 hr. The mRNA levels in the petiole extracts were quantified by RT-PCR. Both sucrose and glucose equally induced strong expression of *Ipoba;CycD3;1* (Fig.4). The expression of *Ipoba;CycD3;2* was also induced strongly by sucrose and to a lesser extent by glucose. Sucrose also induced the expression of *iAGPLI-1*, but glucose did not. The mannitol treatment was used as a control for the osmotic effect of the sugars and shows that the sugar-induced expression of *Ipoba;CycD3;1*, *Ipoba;CycD3;2*, and *iAGPLI-1* was not due to changes in osmolarity. We treated leaf-petiole cuttings with various concentrations of sucrose (0, 50, 100 and 150 mM) to

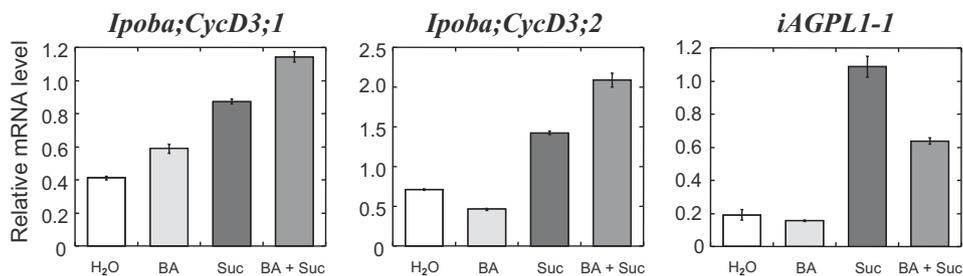


Fig. 6. Steady-state levels of mRNA for *Ipoba;CycD3;1*, *Ipoba;CycD3;2* and *iAGPLI-1* in leaf-petiole cuttings of sweet potato after treatment with sucrose and BA. Total RNA was isolated from the petiole portions of leaf-petiole cuttings that had been treated with 100  $\mu$ M BA (BA), 150 mM sucrose (Suc), 100  $\mu$ M BA and 150 mM Sucrose (BA + Suc) or water (H<sub>2</sub>O) for a day. The mRNA levels of *Ipoba;CycD3;1*, *Ipoba;CycD3;2* and *iAGPLI-1* were normalized relative to 18S rRNA levels. Each value represents the mean  $\pm$  SE of results from three experiments.

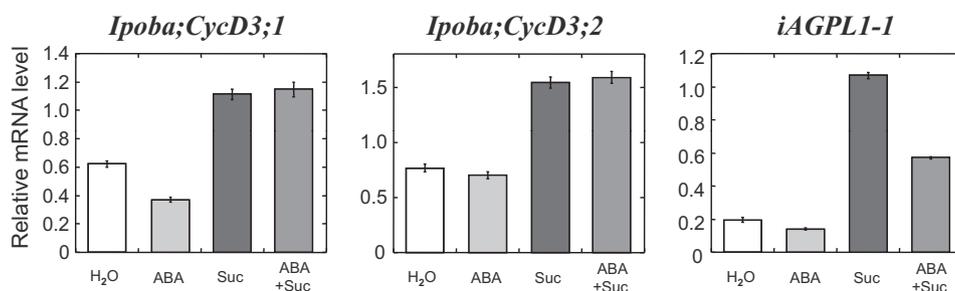


Fig. 7. Steady-state levels of mRNA for *Ipoba;CycD3;1*, *Ipoba;CycD3;2* and *iAGPLI-1* in leaf-petiole cuttings of sweet potato after treatment with sucrose and ABA. Total RNA was isolated from the petiole portions of leaf-petiole cuttings that had been treated with 100  $\mu$ M ABA (ABA), 150 mM sucrose (Suc), 100  $\mu$ M ABA and 150 mM Sucrose (ABA + Suc) or water (H<sub>2</sub>O) for a day. The mRNA levels of *Ipoba;CycD3;1*, *Ipoba;CycD3;2* and *iAGPLI-1* were normalized relative to 18S rRNA levels. Each value represents the mean  $\pm$  SE of results from three experiments.

examine the effect of sucrose concentration on the expression of D3 cyclin and AGPase genes. The expression of *Ipoba;CycD3;1* increased in a dose-dependent manner by treatment with 50 to 100 mM sucrose solution, with the highest expression level obtained with 100 and 150 mM sucrose (Fig. 5). *Ipoba;CycD3;2* expression also increased with increasing sucrose concentration, with 100 mM sucrose being optimal for maximum expression. The expression of *iAGPLI-1* increased in a dose-dependent manner with increasing sucrose concentration up to 150 mM.

##### 5. Hormonal regulation of the expression of D3 cyclin and AGPase genes

The levels of cytokinin and ABA together with sucrose increase in the process of tuberous root formation (Nakatani and Komeichi, 1991; Saitou et al., 1997). Therefore, we examined the effects of the treatment of leaf-petiole cuttings with 100  $\mu$ M BA and 150 mM sucrose, as well as a combination of 100  $\mu$ M BA and 150 mM sucrose. BA induced the expression of *Ipoba;CycD3;1* and enhanced the induction of *Ipoba;CycD3;1* expression by sucrose (Fig. 6). The relative amount of *Ipoba;CycD3;2* mRNA was slightly

reduced by BA, while the transcripts increased in response to BA applied together with sucrose. BA did not significantly affect the expression of *iAGPLI-1*, but repressed the induction of *iAGPLI-1* by sucrose.

We evaluated the effect of ABA on the expression of *Ipoba;CycD3;1*, *Ipoba;CycD3;2* and *iAGPLI-1*. Leaf-petiole cuttings were treated with 100  $\mu$ M ABA, 150 mM sucrose and a combination of 100  $\mu$ M ABA and 150 mM sucrose. ABA reduced the amount of *Ipoba;CycD3;1* mRNA relative to the 18S rRNA (Fig. 7), whereas sucrose antagonized the inhibitory effect of ABA on the expression of *Ipoba;CycD3;1*. ABA did not influence the expression of *Ipoba;CycD3;2*. ABA inhibited the induction of *iAGPLI-1* by sucrose, but did not affect the expression. In the presence of sucrose, ABA application repressed *iAGPLI-1* gene expression similar to the result observed with BA (Fig. 6).

##### Discussion

Petioles function as temporary sink, and the genes coupled with the expression of sink functions of cells are expressed in the petioles, as normally exhibited by cells of tuberous roots in sweet potato (Nakamura et al., 1991). In this study, we investigated the regulation

of expression of genes related to the sink strength of developing roots by sugars and phytohormones using leaf-petiole cuttings, since the developing roots had sugars and phytohormones (Nakatani and Komeichi, 1991; Saitou et al., 1997) and it was difficult to control those amounts.

The level of transcript for *Ipoba;CycD3;1* was highest in the thick root, and was maintained in the tuberous root (Fig. 3). *Ipoba;CycD3;2* gene expression was induced in the thick root, while expression decreased in the tuberous root. In contrast, the expression of *iAGPLI-1* gene increased continuously during tuberous root formation. The endogenous sucrose content of sweet potato roots has been shown to increase with the formation of tuberous roots (Saitou et al., 1997). The expression of the *Ipoba;CycD3;1* and *Ipoba;CycD3;2* genes was enhanced by sucrose at a concentration up to 100 mM (Fig. 5). The expression of the *Ipoba;CycD3;1* and *Ipoba;CycD3;2* genes was maximal with treatment with 100 mM sucrose, whereas 150 mM sucrose did not induce such a high level of *Ipoba;CycD3;2*. On the other hand, sucrose induced the expression of the *iAGPLI-1* gene in a dose-dependent manner up to a concentration of 150 mM. These results indicate that the sucrose concentration regulates the cell division activity and the sink activity during tuberous root development in sweet potato.

Plant D-type cyclins and AGPase play important roles in controlling the cell division and differentiation of storage parenchyma in response to external signals. Sugars are not only the substrate to sustain heterotrophic growth but are also important signals to regulate a variety of genes in higher plants (Koch, 1996). A hexokinase-signaling pathway has been previously characterized for the repression of photosynthetic genes by glucose (Moore et al., 2003). While sucrose induced the expression of *iAGPLI-1*, glucose did not (Fig. 4). This suggests that the hexokinase pathway is not involved in mediating the response of AGPase to a carbon source. On the other hand, the *Ipoba;CycD3;1* and *Ipoba;CycD3;2* genes were induced by both sucrose and glucose. Previous experimental data indicate that the level of hexoses regulate cell division (Weber et al., 1997). These hexoses, which are produced by the action of invertase, act as mitotic stimuli, whereas sucrose induces differentiation and leads to storage product synthesis (Weber et al., 1997). We propose that *CycD3* and AGPase participate in distinct sugar-signaling pathways, and various types of sugar may have several roles to regulate cell division activity and sink activity during tuberous root formation.

*Ipoba;CycD3;1* and *Ipoba;CycD3;2* genes were induced by sucrose and this induction was additively enhanced by BA (Fig. 6). Cytokinin repressed the induction of *iAGPLI-1* by sucrose. Endogenous zeatin riboside (ZR) rapidly increases as well as sucrose at the time of tuberous root formation in sweet potato (Nakatani

and Komeichi, 1991; Saitou et al., 1997). In addition, endogenous ZR is concentrated around the root primary cambium, which plays an important role in storage root formation (Nakatani and Matsuda, 1992). Therefore, enhanced accumulation of sucrose and cytokinin around the root primary cambium is likely to promote cell cycle progression, and repress differentiation to storage parenchyma.

Previous anatomical and physiological studies suggested that ABA promotes the thickening of the tuberous root by activating the meristems on the inside of the primary cambium, especially the secondary cambium (Nakatani and Komeichi, 1991). ABA had no effect on the induction of *Ipoba;CycD3;1* and *Ipoba;CycD3;2* gene expression by sucrose (Fig. 7). In contrast, ABA applied with sucrose ablated the induction in *iAGPLI-1* mRNA level compared with sucrose alone. It seems, therefore, that the presence of sucrose together with ABA represses the expression of *iAGPLI-1* while maintaining the expression of the *CycD3* genes. Their effect would be to promote cell division activity in the secondary cambium of sweet potato.

Exogenous application of sucrose and ABA synergistically regulates the expression of the *OsAPL3* gene, one of the large subunits of AGPase in *Oryza sativa* (Akihiro et al., 2005). Rook et al. (2001) determined that the sucrose and ABA concentrations in leaves also regulate the *Apl3* gene, which encodes the large subunit of AGPase in *Arabidopsis*. Studies of the *Arabidopsis Apl3* promoter suggest that two distinct promoter elements are involved in either sugar (sucrose box3 and SURE1 elements (S3S1)-box) or ABA (coupling element1 (CE1)-element) responsiveness of the *Apl3* gene (Rook et al., 2006). We found that *iAGPLI-1* transcripts decreased in response to sucrose when ABA was also supplemented (Fig. 7). Therefore, it appears that sucrose and ABA alone or in combination differentially regulate *iAGPLI-1*.

## Conclusion

The experimental data presented here suggest that sugars play important roles during tuberous root formation. Our results demonstrate that the sucrose concentration regulates gene expression of *Ipoba;CycD3;1*, *Ipoba;CycD3;2*, and *iAGPLI-1*. Therefore, we conclude that the differential response of these genes to sugars regulates cell division and the sink activity during tuberous root development. Additionally, both cytokinin and ABA interact with elevated levels of sucrose, further promoting the cell division activity. In order to understand this regulation, we need to focus subsequent studies on the mechanism of transcriptional regulation induced by these sugars and phytohormones.

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\* In Japanese with English summary.

\*\* In Japanese with English abstract.