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Apple aminopropyl transferase, MdACL5 interacts with putative elongation factor 1-α and *S*-adenosylmethionine synthase revealed

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Abstract

Several lines of evidence suggest different allocations of the physiological roles of *aminopropyl transferase* genes, *SPMS* and *ACL5* in plants. To get deeper insights into the physiological role of apple *ACL5* (*MdACL5*), we performed yeast two-hybrid (Y2H) assay to identify proteins which interact with MdACL5. After intense screening processes, including the swapping of the bait and prey vectors and *in vitro* coimmunoprecipitation, we identified three MdACL5-interacting proteins: putative translation elongation factor 1A (eEF-1A), putative *S*-adenosyl-L-methionine synthetase (SAMS) and an unknown protein. Results from Y2H and RNA gel blot analysis suggested the involvement of MdACL5 and eEF-1A or SAMS complexes in the plant growth and development of the organized tissues and/or organs.

Keywords: ACL5; Apple (*Malus sylvestris* var. *domestica*); Polyamine; Yeast two-hybrid

Polyamines, namely, putrescine (Put), spermidine (Spd), and spermine (Spm), are aliphatic amines present ubiquitously in all living organisms. Put is biosynthesized either directly from ornithine by ornithine decarboxylase (ODC) or indirectly from arginine by arginine decarboxylase (ADC) [1,2]. Put is further converted into Spd by spermidine synthase (SPDS) with the addition of an aminopropyl moiety provided by decarboxylated *S*-adenosylmethionine (dcSAM), which is catalyzed by *S*-adenosylmethionine decarboxylase (SAMDC) using *S*-adenosylmethionine (SAM) as a substrate. Similarly, Spm is produced from Spd by spermine synthase with the same aminopropyl moiety rendered by dcSAM.

Recent advances in molecular biological techniques, e.g. gene isolation and subsequent gene introgression into plants, enable us to directly verify the physiological roles of polyamines. The involvement of *ADC*, *ODC*, *SAMDC*, and *SPDS* in a variety of important physiological processes such as morphogenesis, growth and biotic/abiotic stress responses has been verified through the analysis of transgenic plants (e.g. [3]). In addition, the genomic sequence data provide information on the regulation of gene expression. For example, an abscisic acid (ABA)-responsive element-related motif and the *cis*-acting element DRE/CRT were found in the 5′-upstream regions of some polyamine biosynthetic genes [4].

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Compared with *ADC*, *ODC*, *SAMDC*, and *SPDS*, the progress in the molecular analysis of *spermine synthase* has lagged far behind. Plant *spermine synthase* was first identified by the analysis of an *Arabidopsis* mutant, *acaulis5*, as a cause gene for its stunted phenotype, designated *ACL5* [5]. Recently, it has been shown that ACL5 is not a spermine synthase but a thermospermine synthase [6]. In addition, another aminopropyl transferase, spermine synthase (*AtSPMS*), previously called *AtSPDS3* due to its high homology to the SPDS group, was also reported [7]. It was shown that the identity between the MdSPMS and MdACL5 is about only 20%. Some evidences indicate that these two genes have different roles in plants. For example, *AtSPMS* and *ACL5* were induced by ABA and auxin, respectively [8]. A T-DNA insertion mutant at *ACL5* revealed a severe defect in stem elongation, like *acl5*, whereas the mutant at *AtSPMS* did not show any obvious morphological changes in spite of a significant decrease in the free and conjugated Spm titer [9]. Furthermore, existence of a metabolon by forming heterodimers SPDS2-SPMS by *in vitro* binding and 'in planta' methods as well as by a yeast two-hybrid (Y2H) system was reported [7]. In order to reveal the actual physiological role of *ACL5*, therefore, we isolated proteins which interact with ACL5 by Y2H assay. Since we have isolated the apple *aminopropyl transferase* gene, *MdACL5* [10], we used it as bait to search for candidate protein(s) from the cDNA library. Based on the putative amino acid sequence similarities and expression patterns of the candidate clones, we suggest the involvement of MdACL5 and eEF-1A or SAMS complexes in the plant growth and development of the organized tissues and/or organs.

Materials and methods

Construction of cDNA library. To construct a cDNA library for Y2H screening, total RNA was extracted by a hot borate method [18] from 'Orin' (*Malus sylvestris* var. *domestica*) young fruits at 19 days after full bloom (DAFB), when *MdACL5* expression exhibited the highest during fruit development [10]. The poly(A)$^+$RNAs were isolated from total RNA using Oligotex^{TM}-dT30 <Super> (TaKaRa, Shiga, Japan), and a cDNA library for Y2H screening (pGADT7-Rec/cDNAs) was constructed using the Matchmaker Library Construction and Screening Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.

Preparation of the bait plasmids and transformed yeast strains. Full-length of *MdACL5* (Accession No. AB204521) and *MdSPMS* (AB204520) coding regions were amplified by PCR using the specific primer pairs with EcoRI/PstI and NcoI/BamHI restriction sites (underlined): Acl5-Eco-F (5′-CCA GAA TTC ATG GGT GAG GCT GTT GAG TTT-3′)/Acl5-Pst-R (5′-CGA CTG CAG TAA ATT CGG TAA GCC ACC C-3′) for *MdACL5* and Spms-Nco-F (5′-CCG CCA TGG AGG ACG GCG CAG GAA GAG G-3′)/Spms-Bam-R (5′-CCC GGA TCC TTC TCG CCG ATT TGT CTT GC-3′) for *MdSPMS*. The PCR products were then digested with the corresponding restriction enzymes before ligation into pGBKT7 (Clontech) using the DNA Ligation kit v2.1 (TaKaRa). The products were named pGBKT7/MdACL5 and pGBKT7/MdSPMS, respectively, and their nucleotide sequences were verified using the BigDye®Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and 3130xl Genetic Analyzer (Applied Biosystems). pGBKT7/MdACL5 and pGBKT7/MdSPMS plasmid were then separately introduced into yeast, strain AH109 (Clontech), using a modified lithium acetate protocol [19]. The transcriptional activation of pGBKT7/MdACL5 or pGBKT7/MdSPMS and the toxicity of MdACL5 or MdSPMS to AH109 were checked according to the manufacturer's instructions.

Yeast two-hybrid screening. Y2H assay was carried out according to the manufacturer's instructions. The co-transformants were plated onto a TDO medium and incubated at 30 °C until colonies appeared (about 5–7 days), and the firm colonies were further screened by TDO containing 5 mM 3-amino-1,2,4-triazole (3-AT) and QDO with or without 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-gal, Wako Ltd., Osaka, Japan) by detecting the initiation of reporter gene transcription (*HIS3*, *ADE2*, and *MEL1*). Finally, the blue colonies were selected as positive clones. Plasmids were extracted from positive clones using a Zymoprep^{TM} yeast plasmid minipreparation kit (Zymo Research, Orange, CA, USA). The insert cDNAs cloned into pGADT7-Rec were amplified by PCR using the primer pair ADseq-F (5′-TAA TAC GAC TCA CTA TAG GGC-3′) and ADseq-R (5′-AGA TGG TGC ACG ATG CAC AG-3′). PCR products were sequenced as described above, and their sequences were checked for homology using the BLASTX program against the GenBank/EMBL/DDBJ non-redundant sequence database. The sequence (MDYF-051), which shared no homology hits with the BLASTX program, was further searched against the data set for plant (all Chrolophyta and Streptophyta) EST sequences of GeneBank dbEST.

Verification of protein–protein interaction by vector swapping. To reconfirm the interaction of proteins in yeast, we performed vector swapping between bait and prey. The plasmids of pGBKT7, *MdACL5* and three pGADT7-Rec/positive candidate clones (MDYF-025, MDYF-051, and MDYF-132) were digested with both EcoRI and BamHI. These fragments were then fractioned on 1.0% agarose gel and recovered using a QIAquick gel extraction kit (QIAGEN, Tokyo, Japan). The *MdACL5* fragment was ligated into the recovered pGADT7-Rec vector with the EcoRI/BamHI site and named pGADT7-Rec/MdACL5. Digested fragments of each positive clone were ligated into the EcoRI/BamHI sites of the pGBKT7 vector and named pGBKT7/positive clones. After checking the nucleotide sequences of these swapped clones, co-transformation of each pGBKT7/positive clone with pGADT7-Rec/MdACL5 was carried out using the methods described above.

Coimmunoprecipitation. Protein–protein interactions observed in yeast were confirmed by *in vitro* approach as well. The proteins were transcribed and translated *in vitro* from the both MdACL5 in a pGBKT7 vector (c-Myc tag) and the selected proteins (MDYF-025, MDYF-051, and MDYF-132) in a pGADT7-Rec vector (HA tag) using TnT® Coupled Reticulocyte Lysate Systems (Promega, Madison, WI, USA) in the presence of Redivue L-[35S]methionine (GE Healthcare Bio-Sciences, NJ, USA). Translation products were analyzed by SDS–PAGE. Coimmunoprecipitation was carried out with the Matchmaker Co-IP Kit according to the manufacture's protocol (Clontech), using anti c-Myc or anti-HA antibodies and protein A beads. Results were analyzed after SDS–PAGE and exposed to an imaging plate (BAS-IP MS 2025, Fuji Film, Tokyo, Japan). Radioactive images were obtained with a scanner (BAS 1800 II, Fuji Film).

Expression analysis of the candidate genes in apple tissues and cells. Young leaves, young shoots and flowers (balloon stage) of the apple cultivar 'Orin' were collected from the experimental farm of the National Institute of Fruit Tree Science (Tsukuba, Japan). Fruits were collected at 19, 61, 103, 145, and 174 DAFB from the Apple Research Center, National Institute of Fruit Tree Science (Morioka, Japan). Apple suspension-cultured cells were established and maintained according to the previous report [20]. The suspension cultures were kept fresh by subculturing three times at 2-week intervals just before use. Suspension cells were harvested at 2, 4, 6, 8, 12, 16, and 20 days after the culture was transferred into a flesh medium for RNA extraction. All samples were immediately frozen by liquid nitrogen and stored at −80 °C to prevent mRNA degradation. Total RNA (8 μg) was electrophoresed on 1.2% agarose gel containing formaldehyde and blotted onto a nylon membrane (Hybond-N, Amersham Biosciences, Piscataway, NJ, USA). Insert DNA fragments of the candidate cDNA clones and *MdACL5* were labeled with DIG-dUTP (Roche Diagnostics, Mannheim, Germany) using primer sets of

ADInsert-F (5′-TTC CAC CCA AGC AGT GGT AT-3′)/ADInsert-R (5′-GAT TCG ATG CCC ACC CTC TA-3′) and BDAcl5-F1 (5′-AAG CGT TTC GGG AAG GTA CT-3′)/BDAcl5-R1 (5′-TTG TTC ATG GTT GCA GAG GA-3′) by PCR, respectively. Hybridization, washing and detection were performed basically according to the method of Kitashiba et al. [10]. The membrane was washed twice in 0.1× SSC and 0.1% SDS at 68 °C for 15 min.

Results and discussion

Screening the binding partner of MdACL5 in apple cDNA library

In order to search for the proteins that interact with MdACL5 in yeast, we carried out Y2H screening using a cDNA library that was constructed from fruit collected 19 DAFB. After ascertaining that only the bait plasmid, pGBKT7/MdACL5, was not able to activate any yeast reporter genes and had no toxicity on yeast cells (data not shown), it was co-transformed with the pGADT7-Rec/cDNA library into AH109 yeast competent cells. Yeast cells in which protein–protein interactions took place were able to survive on the medium SD/-Leu/-Trp/-His (TDO) plate; in contrast, merely co-transformed cells could not grow on the medium TDO but did on the medium SD/-Trp/-Leu (DDO). Consequently, we found five candidate proteins, designated MDYF-025, 037, 051, 124, and 132 (Fig. 1A). These proteins also showed blue color on the SD/-Leu/-Trp/-His/-Ade (QDO)/ X-α-gal plate (data not shown), indicating the high expression of the yeast reporter genes, *HIS3*$^+$, *ADE2*$^+$, and *MEL1*$^+$. The nucleotide sequences of these candidates were subjected to a homology search against the sequence databases using BLASTX, through which we found five kinds of putative genes, including two *translation elongation factor 1A* (*eEF-1A*), two *S-adenosyl-L-methionine synthetase* (*SAMS*) and an unknown one (Table 1). A homology search of the unknown gene (*MDYF-051*) using BLASTN against the EST database did not reveal any counterparts in apple EST collections; however, its homologues were found in the rose, apricot and grape EST collections (data not shown), indicating that *MDYF-051* might not an artifact but a transcribed gene. Based on the sequence similarities and the insert lengths of each duplicated gene, we selected MDYF-025 and 132 as representatives of *eEF-1A* and *SAMS*, respectively. Then, in order to eliminate false-positive reactions, vectors were swapped with each other. These clones grew well on the DDO and TDO plates (Fig. 1B) and showed blue color on the QDO/X-α-gal plate (data not shown), which suggested that MdACL5 and the proteins from the three clones certainly interact in yeast. Protein–protein interactions observed in yeast cells were also confirmed *in vitro* using coimmunoprecipitation (Fig. 2). Another apple aminopropyl transferase, MdSPMS, and each of these three clones were also co-transformed into

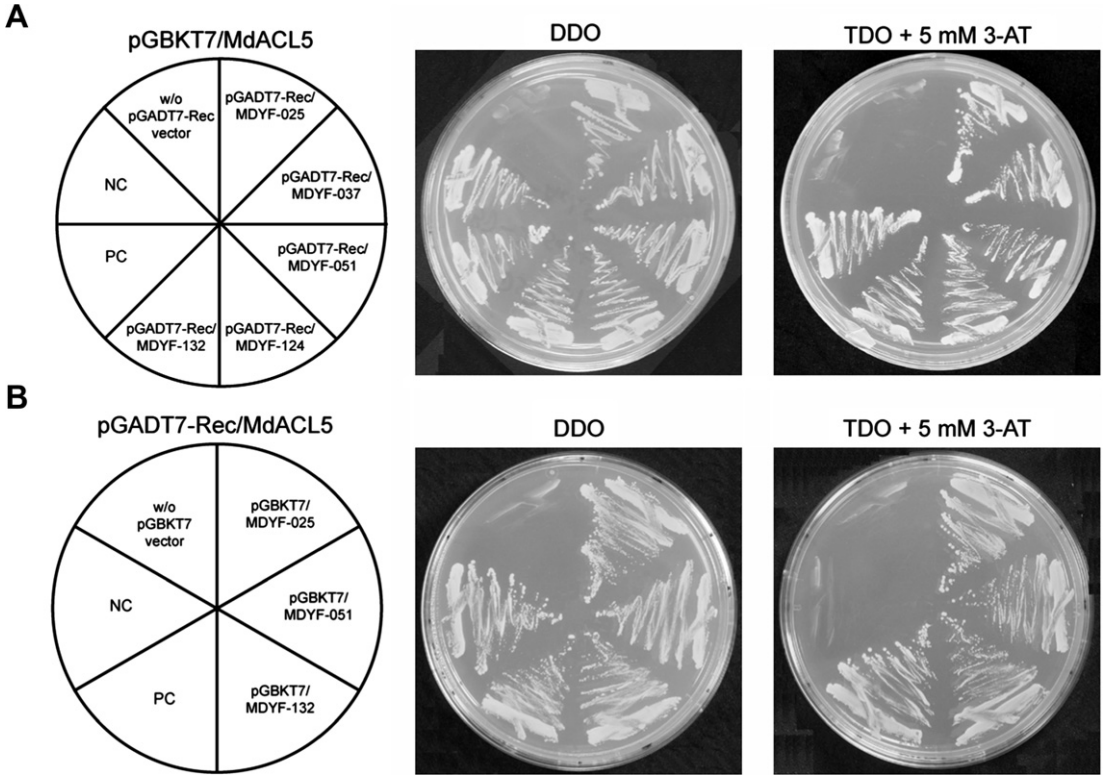

Fig. 1. Interaction of MdACL5 with the proteins by yeast two-hybrid assay (A) and verification of protein–protein interaction by vector swapping (B). In both panels, circles show the arrangement of AH109 yeast cells carrying pGBKT7 or pGADT7-Rec plasmids. Yeast grows on the selected media of SD/-Trp/-Leu (DDO) and SD/-Trp/-Leu/-His (TDO) + 5 mM 3-AT. PC and NC indicate positive (pGADT7-RecT + pGBKT7-53) and: negative control (pGADT7-RecT + pGBKT7-Lam), respectively.

Table 1
Identified interaction protein in apple cDNAs by two-hybrid screening

Clone reference	cDNA size (bp)	Best database match	E-value	Accession No.
MDYF-025	977	Elongation factor 1-α (*Lilium longiflorum;* AAD27590)	2E−104	AB294182
MDYF-037	921	Translation elongation factor 1A-5 (*Gossypium hirsutum;* ABA12221)	2E−104	AB294183
MDYF-051	273	Unknown	—	AB294184
MDYF-124	723	*S*-adenosyl-L-methionine synthetase (*Elaeagnus umbellata;* AAK29410)	3E−94	AB294185
MDYF-132	826	*S*-adenosyl-L-methionine synthetase (*Elaeagnus umbellata;* AAK29410)	2E−98	AB294186

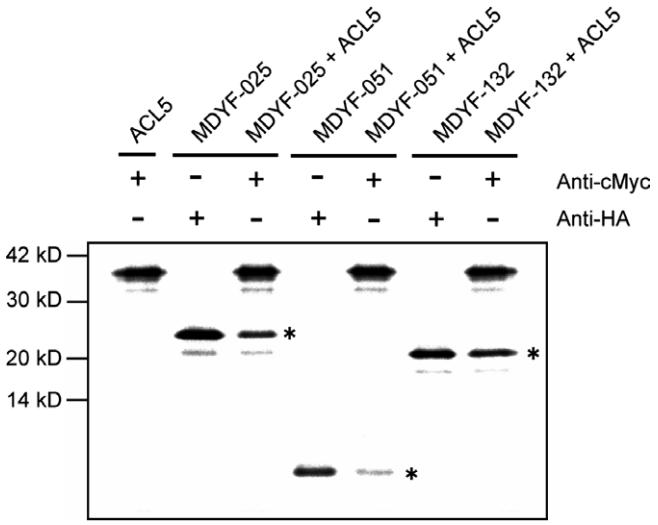

Fig. 2. SDS–PAGE analysis shows that the each selected protein (MDYF-025, putative eEF-1A; MDYF-051, unknown; MDYF-132, putative SAMS) coimmunoprecipitate with MdACL5. MdACL5 and the selected proteins were immunoprecipitated by cMyc-tag monoclonal antibody and HA-tag polyclonal antibody, respectively. For coimmunoprecipitation, the mixture of MdACL5 and the each selected proteins were immunoprecipitated by cMyc-tag monoclonal antibody. Asterisks indicate the each selected protein in the coimmunoprecipitate fraction.

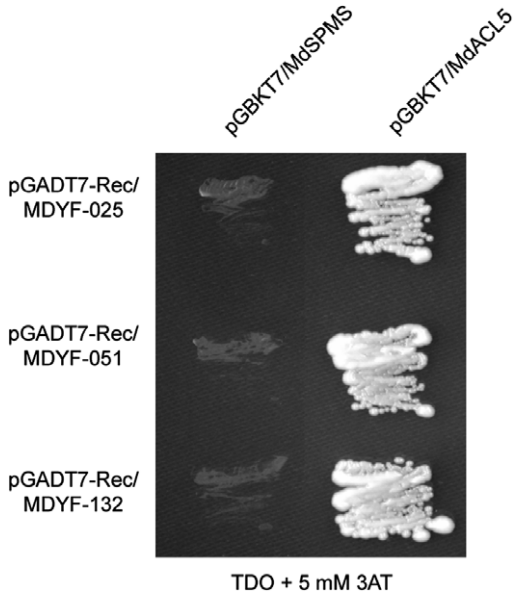

Fig. 3. Interaction of MdSPMS with the selected proteins (MDYF-025, putative eEF-1A; 051, unknown; 132, putative SAMS) in a yeast two-hybrid assay. For positive control, the interactions of MdACL5 and the selected proteins (MDYF-025, putative eEF-1A; 051, unknown; 132, putative SAMS) are shown. In all panels, yeast grows on the selected media of SD/-Trp/-Leu/-His (TDO) + 5 mM 3-AT.

the yeast; however, these transformants could not grow on the TDO plate, indicating no interaction between MdSPMS and the three clones (Fig. 3).

eEF-1A, one of the four subunits composing eukaryotic translation elongation factor-1 (eEF-1), plays an important role in protein synthesis, through catalyzing the binding of aminoacyl-tRNA to the A-site of the ribosome by a GTP-dependent mechanism [11,12]. It has been known that polyamines such as spermidine and spermine can regulate peptide chain elongation [13,14]. Therefore, thermospermine may be also involved in peptide chain elongation possibly through regulating the aminoacyl-tRNA synthesis. If this is the case, interaction of MdACL5 with eEF-1A might be plausible. However, several questions still remain to be clarified, taking into consideration that MdSPMS did not show the interaction with eEF-1A irrespective of the reports on the effectiveness of spermine for protein synthesis [14]. On the other hand, SAMS produces a key molecule, SAM, for both the ethylene and polyamine biosynthetic pathways in plants. Antagonistic relationships of ethylene and polyamine through the balance of SAM were demonstrated [15]. To our knowledge, this is the first report on the relationships between MdACL5 and eEF-1A or SAMS.

Expression levels of MdACL5 and the two selected clones in apple tissues and cell cultures

RNA gel blot analysis was performed with total RNA isolated from different apple tissues using the digoxigenin (DIG) system. Each insert DNA of *MDYF-025* (putative eEF-1A), *MDYF-051* (unknown), and *132* (putative SAMS) plasmids and the *MdACL5* fragment were amplified by PCR, labeled by DIG and then used as a probe. In the vegetative tissues, the *MdACL5* transcript was detected from shoot and young leaf (Fig. 4A). In the reproductive tissues, however, mRNA expression was detected only in young flower buds and fruits at 19 and 61 DAFB (Fig. 4A), in accord with the previous report of Kitashiba et al. [10]. On the other hand, *MDYF-025* (putative eEF-1A), *MDYF-051* (unknown), and *132* (putative SAMS) showed high expression levels not only in the vegetative tissues but also in the reproductive tissues throughout the developmental stages (Fig. 4A). During fruit ripening, the

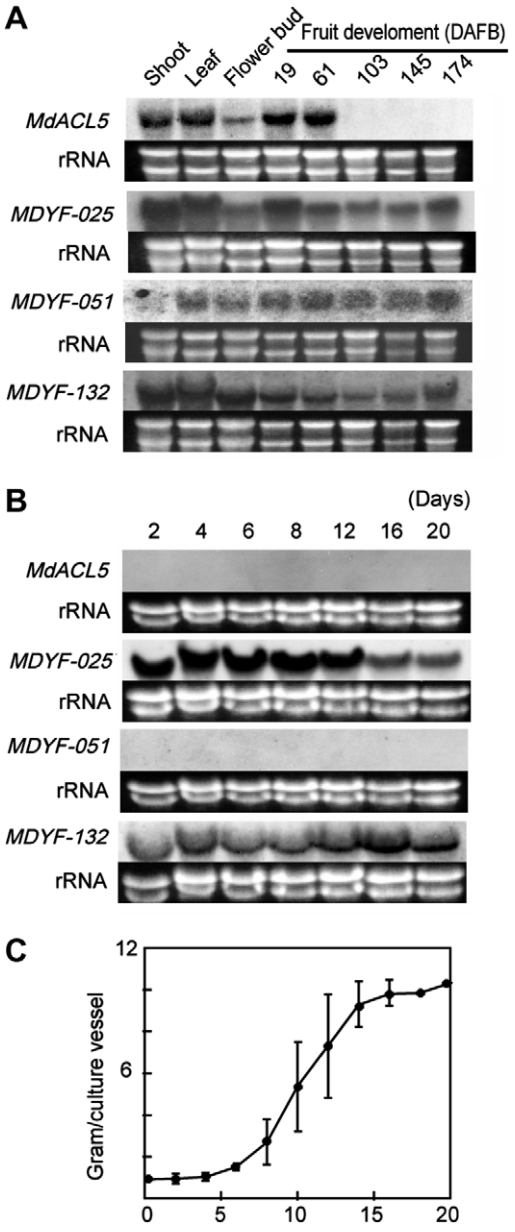

Fig. 4. Expression of *MdACL5*, *MDYF-025* (putative eEF-1A), *MDYF-051* (unknown), and *MDYF-132* (putative SAMS) in various apple tissues and fruits (A) and in apple suspension cultures (B), along with growth curve of suspension cultures (C). Ethidium bromide staining shows the equal loading of rRNA.

expressions of *MDYF-025* (putative eEF-1A), *MDYF-051* (unknown), and *132* (putative SAMS) were high at the early stage and re-increased at the late stage after a temporal decline at the middle stage. We had already shown the interesting expression of *MdACL5* that was limited to systematically organized tissues; no signal was detected in the undifferentiated tissues such as callus and suspension cultures in apple [10]. Thus, we investigated the expressions of *MDYF-025* (putative eEF-1A), *MDYF-051* (unknown), and *132* (putative SAMS) in the apple suspension cultures. *MDYF-051* (unknown) transcript was under the detectable level like *MdACL5*. On the other hand, constant expressions of *MDYF-025* (putative eEF-1A) and *132* (putative SAMS) could be detected throughout the cell culture periods (Fig. 4B). *MDYF-025* (putative eEF-1A) expressed relatively highly at the early and middle stage, the latter of which corresponded to the active cell division and proliferation stage (Fig. 4C). In contrast, *MDYF-132* (putative SAMS) expressed in a reverse manner, with relatively low levels at the middle stage and high levels at the late stage.

It is known that cell division of the fruit occurs only in a limited period after fruit setting, approximately 19–61 DAFB. Therefore, MdACL5 and eEF-1 complex could facilitate the cell development/elongation of plants through the activation of protein synthesis. On the other hand, it has been reported that SAMS activity and its proteins were preferentially expressed in the lignified vascular tissues of plants [16]. Recently, an *Arabidopsis* mutant *thickvein* (*tkv*) that developed thicker veins in leaves and inflorescence stem was revealed to reside in *ACL5*, and its expression was specific to provascular cells involving *ACL5* in a mechanism that defines the boundaries between veins and the non-vein via polar auxin transport [17]. These results suggested the involvement of MdACL5 and SAMS complexes in plant growth and development in organized tissues and/or organs. It is worth noting that the other aminopropyl transferase, spermine synthase (MdSPMS), did not show any interaction with SAMS (Fig. 3) even though the aminopropyl moiety (decarboxylated SAM) derived from SAM, is also necessary for its enzymatic reaction like MdACL5. Instead, it was shown that AtSPMS formed heterodimers with AtSPDS2, while no interaction of ACL5 with SPDSs was found [7]. These previous reports and ours shown here further support the hypothesis that plant *ACL5* has roles different from those of *SPMS*. SAMS is a key enzyme that produces SAM and is related to both ethylene and polyamine synthesis. Further investigation into the physiological roles of MdACL5 is necessary; however, MdACL5 might be more involved in the cascade of the ethylene pathway than is polyamine, in which MdACL5 and eEF-1A or SAMS complexes play important roles in the plant growth and development of the organized tissues and/or organs.

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