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Two types of spermine synthase gene: *MdACL5* and *MdSPMS* are differentially involved in apple fruit development and cell growth

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Abstract

Three cDNAs with high homology to spermine (Spm) synthases in *Arabidopsis* were isolated from apple [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.]. *MdACL5-1* and *MdACL5-2* have high homology with *ACL5* and *MdSPMS* has high homology with *AtSPMS*. The similarity of MdSPMS to spermidine synthases (SPDSs) was higher than that of MdACL5s, despite the fact that both are putative Spm synthases. However, MdSPMS could be discriminated from SPDSs by the presence of several characteristic amino acids, i.e., Val-149, Ser-161, Ala-205, and Val-235, in the decarboxylated *S*-adenosylmethionine (dcSAM)-binding motif of MdSPMS. Both MdACL5-1 and MdSPMS complemented Spm biosynthesis in a yeast mutant deficient in Spm synthase, and ectopic expression of *MdACL5-1* in the *Arabidopsis* dwarf mutant *acl5* allowed recovery of the normal phenotype. RNA gel blot analysis showed that *MdACL5* and *MdSPMS* are differentially expressed in tissues and suspension cells. These results suggest that functional MdACL5 and MdSPMS are independently involved in apple fruit development and cell growth.

Keywords: *ACL5*; Apple (*Malus sylvestris* var. *domestica*); Polyamine; Spermine synthase; SPMS

1. Introduction

Polyamines, i.e., spermidine (Spd), spermine (Spm), and their obligate diamine precursor putrescine (Put), are aliphatic amines produced ubiquitously in living cells and involved in the regulation of many basic cellular processes, such as DNA replication, transcription, translation, cell proliferation, modulation of enzyme activities, and membrane stabilization (Tabor and Tabor, 1984; Walden et al., 1997). In plants, polyamines play important roles in morphogenesis, flower differentiation and initiation, pollen viability, root growth, somatic embryogenesis, anti-senescence, and responses to biotic and abiotic stresses (Evans and Malmberg, 1989; Galston and Sawhney, 1990; Walden et al., 1997; Bouchereau et al., 1999; Pandey et al., 2000; Takahashi et al., 2003). Several lines of evidence also suggest a role of polyamines in fruit development. For example, a high free polyamine titer was observed during the early growth period in apple (Biasi et al., 1988) and peach fruits (Kushad, 1998), and spraying polyamine on flowers increased both fruit set and total yield (Costa and Bagni, 1983). Correspondingly, transcript levels and activity of polyamine biosynthetic enzymes were also investigated during peach fruit development (Ziosi et al., 2003). In addition to studies on fruit development, correlations between polyamine titer and physiological abscission at the fruitlet stage or chilling injury of fruit have also been documented (Evans and Malmberg, 1989; Pandey et al.,

Abbreviations: ADC, arginine decarboxylase; CaMV, cauliflower mosaic virus; DAF, days after full bloom; dcSAM, decarboxylated *S*-adenosylmethionine; DIG, digoxigenin-dUTP; ODC, ornithine decarboxylase; PCA, perchloric acid; Put, putrescine; RACE, rapid amplification of cDNA ends; SAMDC, *S*-adenosylmethionine decarboxylase; Spd, spermidine; SPDS, spermidine synthase; Spm, spermine.

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2000; Aziz et al., 2001; Kondo et al., 2001, 2003). However, the role of polyamines remains a topic of discussion, and interpretations of the results have often been confusing due to the different plant species and/or cultivars studied.

Recent advances in molecular approaches, especially techniques in gene isolation and the creation of transgenic plants, have made it possible to directly evaluate the physiological roles of polyamines in plants. To date, gene isolation and transgene expression experiments have focused mainly on the genes encoding ornithine decarboxylase (*ODC*), arginine decarboxylase (*ADC*), and *S*-adenosylmethionine decarboxylase (*SAMDC*; Kakkar and Sawhney, 2002). Plants carrying one of these transgenes demonstrate a variety of physiological changes, which are accompanied by corresponding changes in polyamine titers (e.g. Capell et al., 2004). Spd synthase (*SPDS*) genes have been also isolated from several plants, such as *Arabidopsis thaliana*, *Pisum sativum*, *Coffea arabica*, *Lycopersicon esculentum*, and *Malus domestica* (Hashimoto et al., 1998; Alabadí and Carbonell, 1999a,b; Hatanaka et al., 1999; Zhang et al., 2003). *SPDS*-overexpressing *Arabidopsis* was shown to tolerate a wide range of stresses, including salinity, chilling, freezing, and ozone exposure (Kasukabe et al., 2004), implying a role for Spd in stress responses.

Several reports have investigated the physiological role of Spm. Borrell et al. (1996) reported the regulation of *ADC* by Spm in osmotically stressed oat leaves. In developing fruits, positive correlation has been observed between Spm and ovary senescence, fruit set, yields and average fruit weight in pea (Carbonell and Navarro, 1989) and in apple (Costa and Bagni, 1983). In tomato, Spm titer at the cell-division stage was higher than at other stages (Egea-Cortines et al., 1993). Spm is also involved in the hypersensitive response of barley to powdery mildew infection (Cowley and Walters, 2002). Recently, Takahashi et al. (2003) reported that Spm was a trigger substance in disease resistance induced via dysfunction of mitochondria. Furthermore, Spm treatment prior to storage reduces chilling injury in fruits such as zucchini squash and mangosteen (Kramer and Wang, 1989; Kondo et al., 2003). However, compared with Put and Spd, the physiological function of Spm is poorly understood, partly due to a lack of insight into its molecular mechanism of action.

The Spm synthase gene was first identified and isolated in the *A. thaliana* null mutant *acl5* (Hanzawa et al., 2000). *ACL5* encodes Spm synthase, and its inactivation causes a defect in the elongation of stem internodes. In addition, a second Spm synthase gene (*AtSPMS*) was characterized by Panicot et al. (2002), Urano et al. (2003), and Imai et al. (2004). AtSPMS was initially designated as *SPDS3*, because of its high similarity to SPDS (ca. 68%); however, it failed to complement the *SPDS* deficiency of a yeast mutant (Hanzawa et al., 2002). Furthermore, Imai et al. (2004) reported a significant decrease in the Spm titer of *spms-1*, which is a T-DNA insertion mutant of *AtSPMS*, compared with the wild type. These reports demonstrate that AtSPMS does not function as a Spd synthase but as a Spm synthase. Therefore, it is apparent that two types of Spm synthase genes, *ACL5* and *AtSPMS* with low amino acid sequence similarity each other, are present in *Arabidopsis*.

We have been studying the involvement of polyamines in fruit development, ripening, and stress responses using apple, one of the most economically important fruit trees worldwide. To elucidate the relationship between Spm and physiological processes, the isolation of genes homologous to *ACL5* and *AtSPMS* is prerequisite, since these two Spm synthase genes are present in *Arabidopsis*. Therefore, as a first step, Spm synthase genes were isolated from apple, and both their tissue-specific expression and function were analyzed.

2. Materials and methods

2.1. Plant materials

Leaves, flowers at the balloon stage, fruits at 19, 61, 103, 145, and 174 days after full bloom (DAF), and suspension cells of 'Orin' apple (*Malus sylvestris* var. *domestica*) were prepared as described by Hao et al. (2005a).

2.2. Isolation of ACL5 homologs from apple

Extraction of total RNA from apple flowers at the balloon stage and synthesis of first-strand cDNA were conducted as described Hao et al. (2005a). Degenerate primers [5′-GARAAGTTTGATATCATMGTKG-3′ (forward) and 5′-ATGWCCAKRAATRAAYCTMGC-3′ (backward)] were designed based on the amino acid sequences KFDIIVG and ARFIHGH in ACL5 from *Arabidopsis* and on *ACL5* homologous expressed sequence tags in other plants. RT-PCR was conducted in a 50 μl reaction mixture containing 1.0 μl first-strand cDNA, 1 μM of each primer, 200 μM dNTPs, and 0.5 U Ex *taq* polymerase (TaKaRa) in 1 × Ex *taq* polymerase buffer using a DNA thermal cycler (Perkin Elmer). RT-PCR conditions were 96 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min (30 cycles). The PCR product was subcloned into the vector pCR 2.1 (Invitrogen). After sequencing, the PCR product was labeled with digoxigenin-dUTP (DIG; Roche Diagnostics) and used as a probe to screen a cDNA library derived from apple flowers at the balloon stage. Samples were hybridized in 5 × SSC, 0.5% blocking reagent (Roche Diagnostics), 0.1% sodium *N*-lauroylsarcosine, and 0.02% SDS at 65 °C. Washing was performed twice in 2 × SSC, 0.1% SDS at room temperature for 5 min and then twice in 0.1 × SSC, 0.1% SDS at 65 °C for 20 min. Signal was detected using CSPD (Roche Diagnostics). Positive plaques were excised as pBluescript clones following the manufacturer's instructions (Stratagene) and sequenced in both strands using the