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Subcellular localization and vacuolar targeting of sorbitol dehydrogenase in apple seed

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ABSTRACT

Sorbitol is the primary photosynthate and translocated carbohydrate in fruit trees of the *Rosaceae* family. NAD^+-dependent sorbitol dehydrogenase (NAD-SDH, EC 1.1.1.14), which mainly catalyzes the oxidation of sorbitol to fructose, plays a key role in regulating sink strength in apple. In this study, we found that apple NAD-SDH was ubiquitously distributed in epidermis, parenchyma, and vascular bundle in developing cotyledon. NAD-SDH was localized in the cytosol, the membranes of endoplasmic reticulum and vesicles, and the vacuolar lumen in the cotyledon at the middle stage of seed development. In contrast, NAD-SDH was mainly distributed in the protein storage vacuoles in cotyledon at the late stage of seed development. Sequence analysis revealed there is a putative signal peptide (SP), also being predicated to be a transmembrane domain, in the middle of proteins of apple NAD-SDH isoforms. To investigate whether the putative internal SP functions in the vacuolar targeting of NAD-SDH, we analyzed the localization of the SP-deletion mutants of MdSDH5 and MdSDH6 (two NAD-SDH isoforms in apple) by the transient expression system in *Arabidopsis* protoplasts. MdSDH5 and MdSDH6 were not localized in the vacuoles after their SPs were deleted, suggesting the internal SP functions in the vacuolar targeting of apple NAD-SDH

1. Introduction

Sorbitol is a major photosynthetic product and phloem-translocated component in the woody *Rosaceae*, important crops like apple (*Malus domestica* Borkh.), pear (*Pyrus communis* L.), and peach (*Prunus persica* (L.) Batsch) [1–3]. Sorbitol accounts for about 80% of the total soluble carbohydrate in apple leaves. In *Rosaceae* plants, sorbitol is synthesized in source organs from glucose-6-phosphate to sorbitol-6-phosphate catalyzed by the NADP-dependent sorbitol-6-phosphate dehydrogenase (S6PDH, EC 1.1.1.200) (also named aldose-6-P-reductase, A6PR) [4,5]. Sorbitol-6-phosphate is further converted to sorbitol by sorbitol-6-phosphate phosphatase (SorPP, EC 3.1.3.50) [6]. It has been reported that NADP-dependent sorbitol-6-phosphate dehydrogenase was localized in chloroplasts and the cytosol of protoplasts from apple cotyledons, which means sorbitol was synthesized in the chloroplast and cytosol [7,8]. Sorbitol was detected in the cytosol, chloroplasts, and vacuoles of peach leaves [9]. Sorbitol concentration in cytosol was similar to that in the vacuole, and the highest level of sorbitol was found in the chloroplast. However, most of the sorbitol was in the vacuole because of the larger volume of the vacuole (occupying 68% of the total mesophyll cells) [9].

Sorbitol is converted to fructose mainly catalyzed by NAD^+-dependent sorbitol dehydrogenase (NAD-SDH, EC 1.1.1.14) in apple [10–12]. Sorbitol accounts for about 80% of the total soluble carbohydrate in apple leaves, but only 3–8% of that accumulated in the fruit. Sorbitol imported into apple fruit is not stored but metabolized rapidly after unloading by NAD-SDH [13]. More than ten full-length cDNAs of *NAD-SDH* have been isolated from apple, including *MdSDH5* and *MdSDH6*, and their deduced amino acid sequences have several conserved domains, such as a zinc-containing alcohol dehydrogenase signature, a structural zinc binding site, a NAD-binding pocket, and a catalytic zinc binding site [13–16]. The expression pattern of *MdSDH* (*NAD-SDH* in *Malus domestica*) genes is tissue specific and developmental stage-dependent [14,15]. The expressions of *MdSDH2*, *MdSDH3* and *MdSDH4* are restricted in sink tissues, such as young leaves, stems, roots, and fruits, but *MdSDH1* is highly expressed in source organs (mature leaves) besides sink tissues [14]. In apple fruit, *SDH1* and *SDH3* are expressed in both seed and cortex tissues, while the expression of *SDH2* is limited to cortex, and *SDH6* and *SDH9* are only expressed in seed [15].

NAD-SDH protein is ubiquitously distributed in the tissues of apple leaf and fruit [16]. Moreover, the subcellular localizations of NAD-SDH in the fruits and leaves are multiple and different. In apple

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leaves where sorbitol is synthesized, NAD-SDH is mainly localized in the cytosol, chloroplasts, and vacuoles. But in the fruit flesh in which sorbitol is utilized, NAD-SDH is localized in the cytosol and chloroplasts, and not in the vacuoles [16]. The different and multiple intracellular localizations of NAD-SDH in different tissues and organs indicate that this enzyme may be very important in regulation sorbitol metabolism to keep the osmotic balance in cells, for sorbitol is an osmotic solute. However, little is currently known about the mechanisms of the targeting and translocation of NAD-SDH. NAD-SDH activity in the seeds was found to be much higher than that in the cortex tissues of fruits during the early developmental stages of apple [15]. It suggests that this enzyme plays a more important role in sorbitol metabolism in the seed development. Whetter and Taper reported that sorbitol is present in apple seeds at the initial germinating stages. But it is unknown where the functional site of NAD-SDH is in cotyledon cells and whether the sorbitol is accumulated in seed for seed germination [17].

In our current study, we investigated the distribution and subcellular localization of NAD-SDH in apple cotyledon using the antibodies which its high specificity had been previously demonstrated. We found NAD-SDH was ubiquitously distributed with subcellular multi-localization in developing cotyledon. Sorbitol imported into apple seed is not stored but metabolized rapidly after unloading by NAD-SDH. Moreover, our data suggest that NAD-SDH is imported into the vacuoles mediated by 18 amino-acid residues as an internal SP. The NAD-SDH localized in the seed protein storage vacuoles may play function by being involved in sorbitol metabolism at the early stage of seed germination.

2. Materials and methods

2.1. Plant materials

Apple (*M. domestica* Borkh. cv. Starkrimson) seeds were collected at 8, 10 and 12 weeks after full bloom (AFB). Samples were picked for immediate use or frozen in liquid nitrogen and kept at −80 °C. For immunohistochemistry and subcellular immunogold labeling experiments, samples were fixed immediately after harvest. Preparation, purification and usage of polyclonal antibodies against the NAD-SDH of apple were according to the description by Wang et al. [16].

2.2. Reverse transcription-polymerase chain reaction analysis

Reverse transcription-polymerase chain reaction (RT-PCR) was used to analyze the expression of *MdSDH5* (GenBank accession No. AY849315) and *MdSDH6* (GenBank accession No. AY849316) we cloned from apple in the transcription level in the seeds. The specific primers are as follows: forward primer for *MdSDH5* 5′-ACTATTTACTCGCAGCCTGA-3′, reverse primer for *MdSDH5* 5′-GAATACCAACACTTAAGGGC-3′, forward primer for *MdSDH6* 5′- CGTGTATTCTGTGTCTTCTGTG-3′, and reverse primer for *MdSDH6* 5′-CGGAGATCATGGCTTCTTTAAT-3′. *EF-1a*, an elongation factor of the apple (GenBank accession No. AJ223969), was selected as the control gene. The forward and reverse primers of *EF-1a* are 5′-ATTGTGGTCATTGGYCAYGT-3′ and 5′-CCTATCTTGTAVACATCCTG-3′).

2.3. Immunohistochemistry

The anti-NAD-SDH rabbit polyclonal antibodies we used here had been purified by antigen affinity and their high specificity had been demonstrated in our previous work [16]. The cotyledons were cut into small cubes (about 2–10 mm^3) and were immediately fixed in 4% pre-cooled paraformaldehyde solution (100 mM PBS, pH 7.0) overnight at 4 °C. The fixed samples were dehydrated in a graded ethanol series, replaced with xylene, and embedded in paraffin. Sections made by microtome (6–10 mm thick) were pasted onto slide glasses pretreated with 1% poly-L-lysine solution. The sections were deparaffinized in xylene and rehydrated in serial ethanol rinse (100%, 70%, 50%, and 30% ethanol in PBS). After washing with PBS, the sections were blocked overnight at 4 °C and were incubated with purified anti-NAD-SDH rabbit antiserum solution for 2 h at 37 °C. Followed by another washing in PBS, the samples were incubated in goat anti-rabbit IgG-fluorescein isothiocyanate (FITC) antibodies (diluted 100-fold in PBS solution) for 1 h at 37 °C. The immune signal was detected by Zeiss LSM 510 confocal laser scanning microscope.

2.4. Transmission electron microscopy and immunogold labeling

Ultrathin sections were prepared and immunogold labeling was done essentially as described by McCartney et al. [18]. The cotyledons were cut into small cubes immediately after isolation from fruits (about 2 mm^3), then fixed with a solution of 4% (w/v) paraformaldehyde in 100 mM pre-cooled phosphate buffer (pH 7.2), and incubated for 6 h at 4 °C. After washing with phosphate buffer, the samples were post-fixed overnight in 0.1% (w/v) OsO_4 at 4 °C, then rinsed with the pre-cooled phosphate buffer, and dehydrated through a graded ethanol series. After infiltration for 48 h with Spurr epoxy resin at 4 °C, the samples were polymerized at 58 °C for 12 h, and cut into ultrathin sections. It had been proven that heating at 58 °C for 12 h did not destroy the immunogenicity of NAD-SDH in our previously work [16].

The rabbit polyclonal antiserum against apple NAD-SDH we used here had been affinity-purified and evaluated. It had high specificity to NAD-SDH of apple and it had been used to study the subcelluar localization of NAD-SDH in apple fruits [16]. The sections were etched with 560 mM sodium metaperiodate for 50 min, and 0.1 M HCl for 30 min. After washing with Tris-buffered saline Tween-20 buffer (TBST) (10 mM Tris (pH 7.4), 500 mM NaCl and 0.3% Tween 20) for 5 min, the sections were incubated in TBST buffer containing 2% BSA for 1 h. After washing with TBST buffer, the treated sections were incubated in purified anti-NAD-SDH rabbit antibodies (diluted in TBST buffer, containing 0.1% BSA) at room temperature for 3 h. After extensive washing with TBST buffer containing 2% BSA, the sections were incubated in goat anti-rabbit IgG antibodies conjugated with 10 nm gold (diluted in TBST buffer, containing 0.1% BSA) for 1 h at 37 °C. The sections were rinsed consecutively with TBST containing 2% BSA, TBST buffer, and double-distilled water, and then stained with uranyl acetate. After washed with double-distilled water, the sections were examined under electron microscope.

2.5. Sugar analysis

The soluble sugars and sorbitol were detected of apple seeds at the middle stage of seed development (8 weeks after full bloom (AFB)) and the late stage of development (12 weeks AFB). The sugar extracts were obtained from 100 to 200 mg of cotyledons. After drying at 80 °C and grinding, the samples were treated in 20 ml 80% aqueous ethanol at 75 °C for 30 min. The extracts, after condensation and centrifugation, were used for sugar analysis with high-performance liquid chromatography (HPLC). Samples were subjected to HPLC under following conditions: instrument, Waters 966; analytical column, Sugar Pak; column temp, 90 °C; solvent system, aqueous solution of 0.0001 M Ca-EDTA; flow rate, 0.5 ml/min; injection volume, 5 μl; detectors, differential refractive index detector. The 0.15% sucrose, and 0.1% fructose, glucose, mannitol and sorbitol were used as standards to evaluate the accuracy.