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ORIGINAL PAPER

Genomic Structure, Sub-Cellular Localization, and Promoter Analysis of the Gene Encoding Sorbitol-6-Phosphate Dehydrogenase from Apple

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Abstract Sorbitol is the primary photosynthetic product and the translocatable and storage carbohydrate in apple (*Malus domestica*) and other fruit tree species within the Rosaceae family. Sorbitol-6-phosphate dehydrogenase (S6PDH, EC 1.1.1.200) is the key enzyme in the biosynthesis of sorbitol. In this study, we isolated two full-length genomic sequences for S6PDH from "Gala" apple. The two sequences have same six exons and first two introns, but the sizes of their last three introns are different. The two sequences were mapped to the same loci on chromosome 10. Immunogold electron microscopy analysis demonstrates that the S6PDH is localized mainly in leaf cytosol and chloroplasts. We also isolated and analyzed the promoter region of *S6PDH* and constructed a series of promoter deletion derivatives with β-glucuronidase (GUS) gene to identify the upstream region of the S6PDH gene required for promoter activity. The GUS activity in *Agrobacterium*-mediated transient transformation of tobacco leaves reveals that the -1719 region is more important for gene expression contrasting with other regions in the S6PDH promoter. The promoter region can be induced by cold, dark, and abscisic acid treatment.

Keywords Apple · Sorbitol-6-phosphate dehydrogenase (S6PDH) · Genomic structure · Sub-cellular localization · Promoter analysis

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Introduction

Sorbitol, a sugar alcohol, is commonly found among the woody members of Rosaceae, including *Malus*, *Prunus*, *Pyrus*, and *Eriobotrya* (Bieleski 1982; Soria-Guerra et al. 2011). Besides being the preferred sugar for total photosynthesis products (Bieleski 1982; Loescher 1987), it is the primary form of translocatable and storage carbohydrates in those plants (Zhou et al. 2003). Sorbitol may also serve as an osmoprotectant and be involved in providing tolerances against abiotic and biotic stresses. It plays a key role in osmotic adjustment under drought (Lo Biancoa et al. 2000; Kobashi et al. 2000; Zhang et al. 2010; Li et al. 2011) and cold conditions and NaCl hardiness (Kanayama et al. 2006). Sorbitol is also involved in the tolerance to boron deficiency (Brown and Hu 1996; Brown et al. 1999) and the resistance of disease (Suleman and Steiner 1994).

Sorbitol synthesis shares a common hexose phosphate pool with sucrose production in cytosol. In source leaves, glucose-6-phosphate is converted to sorbitol-6-phosphate by sorbitol-6-phosphate dehydrogenase (S6PDH, EC 1.1.1.200, also named aldose-6-phosphate reductase) (Kanayama et al. 1992). Afterward, sorbitol-6-phosphate is used to form sorbitol via sorbitol-6-phosphatase (EC 3.1.3.50) by dephosphorylation (Zhou et al. 2003). The reaction catalyzed by S6PDH is the key regulatory step in sorbitol synthesis (Cheng et al. 2005). The majority of sorbitol degradation depends on the presence of NAD^+-sorbitol dehydrogenase (EC 1.1.1.14), which catalyzes sorbitol by converting it to fructose in Rosaceae sink tissue (Oura et al. 2000).

Sorbitol-6-phosphate dehydrogenase is believed to play a critical role in the biosynthesis of sorbitol in the members of

Rosaceae (Hirai 1981; Tao et al. 1995; Cheng et al. 2005). It has been purified and well characterized from loquat and apple (Yamaki 1980; Hirai 1981). The cDNA that encodes for this enzyme has been cloned from apple (Kanayama et al. 1992). In its leaves, S6PDH activity is closely related to the transition from sink to source (Loescher et al. 1982). The expression pattern for the gene encoding S6PDH is similar to that for the enzyme activity (Sakanishi et al. 1998). It is reported that the expression of S6PDH gene in apple and peach could be enhanced by abscisic acid, low temperature, high salinity, and drought (Kanayama et al. 2006; Lo Bianco et al. 2000; Cui et al. 2004). Apple *S6PDH* has been transformed into other plant species such as tobacco and persimmon (Tao et al. 1995; Sheveleva et al. 1998; Gao et al. 2001) to increase their sorbitol content and then their osmotic tolerance. This gene has also been transformed into apple in both sense and antisense orientations to improve our understanding of the relationship between sorbitol and sucrose (Kanamarua et al. 2004; Cheng et al. 2005; Teo et al. 2006; Zhou et al. 2006).

Although relatively abundant literatures on S6PDH are available, information is scarce on its molecular characterization, e.g., genomic structure, intercellular localization, and promoter. The research, reported herein, details the results of genomic structure, sub-cellular localization, and promoter of the S6PDH. Our objective is to gain some new information on the characterization of *S6PDH* and the regulating mechanism of sorbitol production in apple.

Materials and Methods

Plant Materials

Two-year-old apple plants (*Malus domestica* Borkh. cv. Gala), grafted onto rootstock *M. sieversii* (Ledeb.) Roem., were grown in pots at the experimental orchard of the Northwest A&F University in Yangling, China. Standard horticultural practices were followed for disease and pest control. The mature and healthy leaves were sampled and immediately frozen in liquid nitrogen and stored at −72°C.

Six-week-old *Nicotiana tabacum* cv. NC89 plants were grown in an environmental growth chamber under controlled conditions of 16-h light (12,000 lux)/8-h dark cycle, 65% relative humidity, and a 25°C (day)/21°C (night) temperature cycle.

RNA and DNA Extractions

Total RNA was extracted according to a modified cetyltrimethylammonium bromide (CTAB) method described by Chang et al. (1993). Prior to reverse-transcription, RNase-free DNAse I (Invitrogen, USA) was used to remove DNA contamination, according to the manufacturer's instructions. Genomic DNA was extracted from the leaves by a CTAB-based protocol (Dellaporta et al. 1983).

Cloning of *MdS6PDH* cDNA and Sequence Analysis

Based on the reported apple S6PDH sequences (D11080), we used Primers S6PDH1 and S6PDH2 (Table 1) to amplify the open reading frame (ORF) of the full-length cDNA. First strand cDNA was synthesized with a RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas). PCR was performed with rTaq DNA Polymerase (TaKaRa), applying the manufacturer's recommended reaction conditions. Products were gel-purified and cloned into the pMD18-T cloning vector. Afterward, positive clones were sequenced with an ABI 3730 sequencer.

Cloning and Analysis of the *S6PDH* Genomic Sequence

Primers S6PDH1 and S6PDH2 were also used to amplify the genomic sequence of DNA templates from *M. domestica* "Gala". Fragments were amplified using LA Taq DNA Polymerase (TaKaRa) according to the manufacturer's recommendations. PCR products were ligated to pMD-18 (TaKaRa) and sequenced.

Expression of Apple *S6PDH* in *Escherichia Coli* and Antiserum Preparation

Full-length *S6PDH* cDNA was amplified with primers for S6PDH5 and S6PDH6 (Table 1) from the vector with *MdS6PDH* gene. *Bam*HI and *Sal*I sites were incorporated into the forward and reverse primers, respectively. After digestion with *Bam*HI/*Sal*I, the PCR product was cloned into vector pET 32a(+) (Novagen). The ligated products were used to transform BL21 (DE3)-competent cells. A recombinant plasmid, pET–S6PDH, was confirmed by restriction enzyme-digestion and sequencing. Competent cells harboring pET–S6PDH were cultured and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside. Cells were pelleted and lysed in a 2× sample buffer of 0.1 M Tris-Cl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 0.1 M DTT. The cell extract was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The recombinant His-tagged proteins were purified by nickel affinity chromatography according to the manufacturer's protocol (Bio-Rad) and were analyzed by SDS-PAGE.

The purified fusion protein (10 mg) was used for standard immunization protocols in rabbits, from which the polyclonal antiserum was collected. Afterward, the IgG fraction (anti-GluGgt) of the antiserum was purified on a protein A-agarose column based on instructions from the

Table 1 Primers used with apple S6PDH genes

Primer	Sequence
S6PDH1	5′-CCGCTCTAGAACTAGTGGACG-3′
S6PDH2	5′-TCGCTTCCGTTGAACAGCC-3′
S6PDH5	5′-CGCGGATCCGGAGAGTGAGAAAACATG-3′
S6PDH6	5′-CGCGTCGACTCGAAGGTTTTTGAATGG-3′
Spromoter1	5′-CCCTTTCTTTGCGGTTGAATAT-3′
Spromoter2	5′-AAACGAGGAGGTGAGGGATGGA-3′
Spromoter3	5′-TCGTCCTTCTCCAGACGCCAAA-3′
SpromoterS1	5′-CGCGTCGACAAACGAGGAGGTGAGGGA-3′
SpromoterS2	5′-CGCGTCGACAAATCGTTTGTGCGCATGA-3′
SpromoterS3	5′-CGCGTCGACAAACAATGGGGAAAGAAAGG-3′
SpromoterS4	5′-CGCGTCGACATGAGCATGAATTGGGAGTG-3′
SpromoterS5	5′-CGCGTCGACCATATTTTTGTGCGCTTTTAC-3′
SpromoterS6	5′-CGCGTCGACAACGTCACCGGACAAAATTA-3′
SpromoterA	5′-TCCCCCGGGGTTTTCTCACTCTCCAAACTCTC-3′

s/S sense, *a/A* antisense

supplier (GE Healthcare BioSciences, Uppsala, Sweden). Pre-immune serum was obtained from the rabbits before immunization.

Sub-Cellular Immunogold Labeling

We followed the method of sub-cellular immunogold labeling essentially as described by Zhang et al. (2004) and Fan et al. (2009). The sections were examined with a JEM-100S electron microscope. Specificity and reliability of the immunogold labeling were tested by two negative controls. In the first, rabbit pre-immune serum, rather than rabbit antiserum, was used to test the specificity of the antiserum. In the second control, the antiserum was omitted to test for possible nonspecific labeling of the goat anti-rabbit IgG antibody–gold conjugate. More than three repetitions of these experiments were conducted for each sample.

Promoter Isolation and *Cis*-Regulatory Element Prediction

We isolated the *S6PDH* promoter with a Genome Walking Kit (TaKaRa), according to the manufacturer's protocol, using Primers Spromoter1, Spromoter2, and Spromoter3 (Table 1). Products were gel-purified and cloned into the pMD18-T cloning vector. Afterward, positive clones were sequenced with an ABI 3730 sequencer. Their sequences were analyzed via plant *cis*-acting regulatory DNA elements (PLACE) and PlantCARE databases (Higo et al. 1999; Lescot et al. 2002).

Construction of Promoter::GUS Fusion Vectors

A series of nested 5′ deletions of S6PDH promoter fragments were generated using PCR amplification from pMD-Spromoter. Forward primers, SpromoterS1–S6 (Table 1) were designed to correspond to the -2396, -2122, -1719, -1066, -646, and -251 sequences of the S6PDH promoter, and the reverse primer SpromoterA (Table 1) was located in the 3′ end of the promoter. *Sma*I and *Sal*I sites were incorporated into the forward and reverse primers, respectively. After digestion with *Sma*I/*Sal*I, the PCR product was cloned into vector *pC0390GUS* (Xu et al. 2010) immediately upstream of the ATG start codon for β-glucuronidase. These new *S6PDH* promoter constructs, verified by sequencing, were introduced into *Agrobacterium* strain EHA105. We used CaMV35S::GUS (*pC35SGUS*) as the positive control and *pC0390GUS* as the negative (Xu et al. 2010).

Agrobacterium-Mediated Transient Assay and *GUS* Activity Analysis in Tobacco Plants

Agrobacterium-mediated transient assays of tobacco leaves were performed as described previously (Sparkes et al. 2006). The fully expanded leaves that were still attached to the intact tobacco plants were conducted by this protocol. Agro-infiltrated leaf samples from each treatment site were collected for each construct to determine β-glucuronidase (GUS) activity.

Quantitative GUS assays were performed as described previously (Jefferson 1987). They were carried out using 4-methyl umbelliferyl glucuronide (Sigma–Aldrich China) as substrate. Fluorescence of the methyl umbelliferone products was quantified using a Hitachi 850 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The total concentration of protein extracts from the tested samples was normalized through a protocol as described previously (Bradford 1976). GUS activity was expressed as nanomolar of 4-methylumbelliferone (4-MU, Sigma–Aldrich) generated per minute per milligram of soluble proteins.

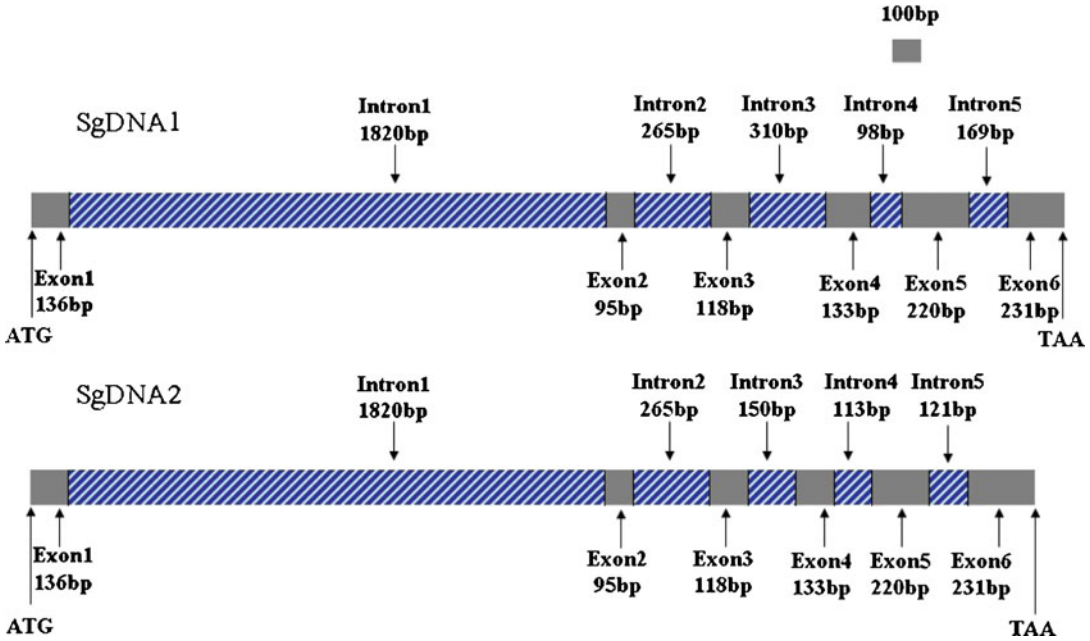

Fig. 1 Structural organization of apple *S6PDH*. Exons are indicated as *black boxes*; introns, as *oblique line boxes*. *Numbers* indicate lengths of each exon and intron

Abiotic Treatments for Agro-Infiltrated Tobacco Plants

Cold, dark, and abscisic acid (ABA) treatment were conducted on tobacco 48 h after agro-infiltration. Cold treatment of agro-infiltrated plants was conducted at 4°C in an incubator for 5 h and of infiltrated plants at 28°C which were used as controls. Dark treatment of agro-infiltrated plants was conducted at 28°C under dark for 5 h and of control plants at 28°C under light (200 μmol m^{-2} s^{-1}). For ABA treatment, agro-infiltrated plants were sprayed with 1 mM ABA for 48 h, and controls were sprayed with sterile water.

Results

Isolation and Analysis of Full-Length Apple *S6PDH* cDNA

Full-length *S6PDH* cDNA was isolated from mature apple leaves. The amplified cDNA fragment, named *MdS6PDH*, was about 1,080 bp long. This gene has been submitted to the GenBank database with accession number AY786539. The cDNA contains a 79-bp 5′-untranslated region (UTR), a 933-bp ORF, and a 68-bp 3′-UTR. The ORF of *MdS6PDH* encodes 310 amino acids. *MdS6PDH* showed 98% and 100% identity in their nucleotide sequence and amino acid sequence levels, respectively, with the reported apple S6PDH (GenBank entry D11080). *MdS6PDH* also showed 80–100% identity with 16 S6PDH or S6PDH-like genes in the apple genome (Velasco et al. 2010).

Genomic Structure and Chromosomal Localization of the Apple *S6PDH* Gene

PCR amplification of the full-length *S6PDH* gDNA produced two different-sized fragments — SgDNA1 (3595 bp, JF764598) and SgDNA2 (3402 bp, JF764599). Alignment of these two gDNA sequences with the cDNA sequence of *MdS6PDH* (AY786539) revealed that each comprises six exons and five introns (Fig. 1). The consensus sequences GT and AG were found at the 5′ and 3′ borders of all introns, which strictly follow the convention for "GT–AG" splicing sites of eukaryotic introns, as proposed by Breathnach and Chambon (1981).

Sequence alignment analysis revealed that the six exons and the first two introns of SgDNA1 and SgDNA2 were 100% identical; therefore, the deduced amino acid sequences of cDNAs for SgDNA1 and SgDNA2 were the same. However, the two sequences differed in the size of their last three introns (Fig. 1). Blast analysis indicated that SgDNA2 was 99% identical with the reported apple S6PDH gDNA (AF057134). To further ascertain the differences in the two gDNAs, the sequence of the last three introns were compared (Fig. 2). Intron 3,intron 4, and intron 5 of SgDNA1 are 32.8%, 74.34%, and 33.73% homologous to those of SgDNA2, respectively. The results indicated that the variation in the last three introns was rooted in base deletion and insertion.

Homology searches of the genome sequence of *M. domestic* cv. Golden Delicious (http://genomics.research.iasma.it/index.html) were conducted with the two cloned S6PDH nucleotide sequences. The genome analysis

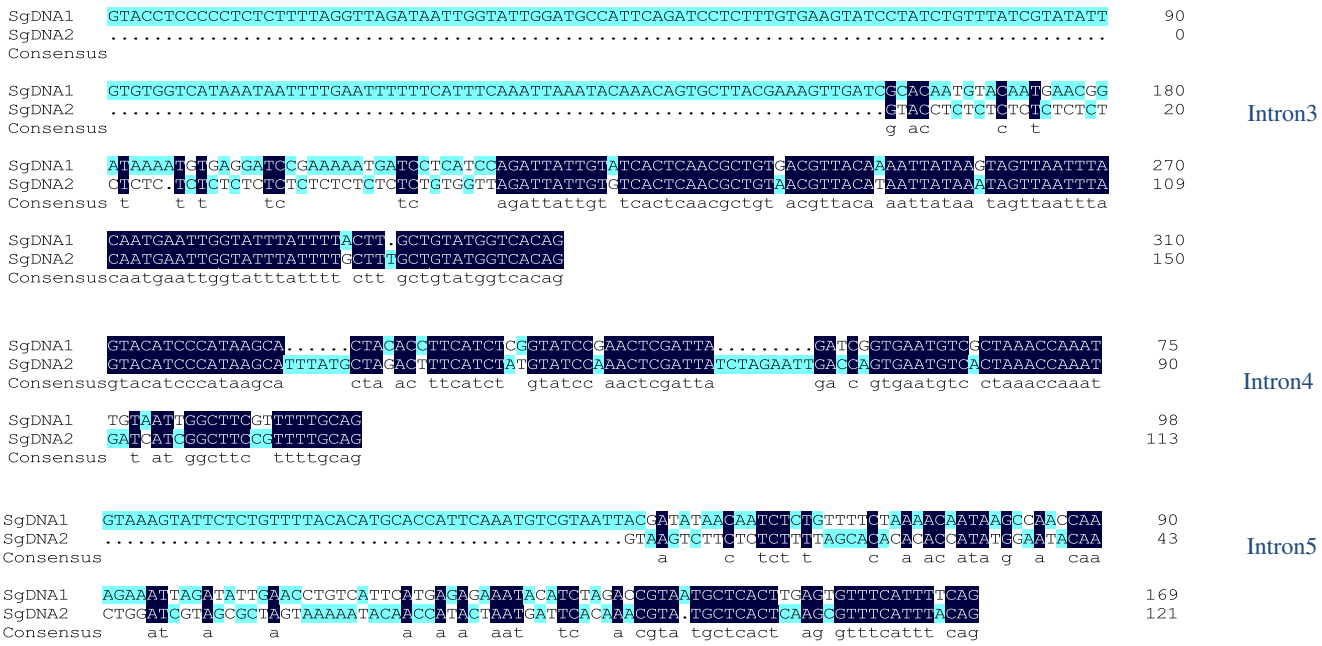

Fig. 2 Comparison of the last three introns of SgDNA1 and SgDNA2

revealed that SgDNA1 and SgDNA2 had high sequence similarity (97.1% and 98.65%) from 11,175,634 bp to 11,179,027 bp in chromosome 10. The present results suggested that SgDNA1 and SgDNA2 were mapped in the same loci, and both existed as alleles.

Antibody Preparation and Sub-Cellular Immunogold-Labeling of *MdS6PDH*

To study the distribution and sub-cellular localization of S6PDH proteins in apple leaves, we selected the full-

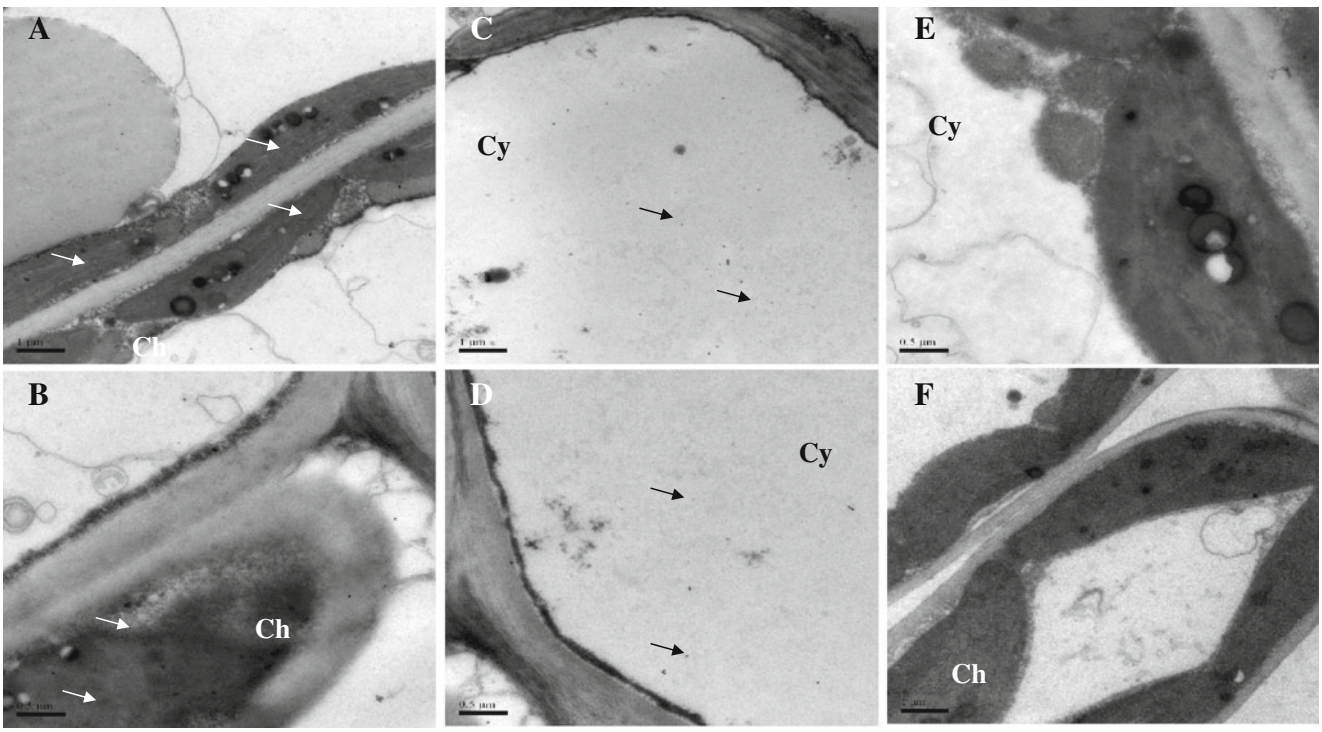

Fig. 3 Transmission electron microscopy result of apple leaf mesophyll cells with anti-MdS6PDH serum and goat anti-rabbit IgG antibody conjugated with 10 nm gold. Particles are distributed mainly in chloroplast (**a** and **b**) and cytosol (**c** and **d**). No immunogold particles were found in pre-immune serum control (**e**) or antiserum omission control (**f**). *Ch* chloroplast; *Cy* cytosol

length 310 amino acids of *MdS6PDH* for antibody preparation. Only one signal was detected by immunoblotting crude protein preparations from the leaves with the MdS6PDH antiserum (data not shown), which indicates that the anti-MdS6PDH serum was recognized S6PDH in apple.

Tissue sections from mature leaves were analyzed with the anti-MdS6PDH serum and an immunogold-labeling secondary antibody. Using transmission electron microscopy of cells within mature tissues, we observed gold particles in both the chloroplast (Fig. 3a, b) and cytoplasm (Fig. 3c, d). No label was seen in other organelles. In experiments to assess the reliability of this immuno-labeling procedure, the control sections displayed no labeling either (Fig. 3e, f).

Isolation of Apple *S6PDH* Promoter Sequences and Characterization Analysis

After isolating the *S6PDH* promoter by PCR and a Genome Walking Kit, we detected a 2.4-kb fragment of product. This promoter, JF827272, was 2,396 bp long (Fig. 4). Its sequence was analyzed for the presence of *cis*-acting elements involved in regulating *S6PDH* expression. PLACE and

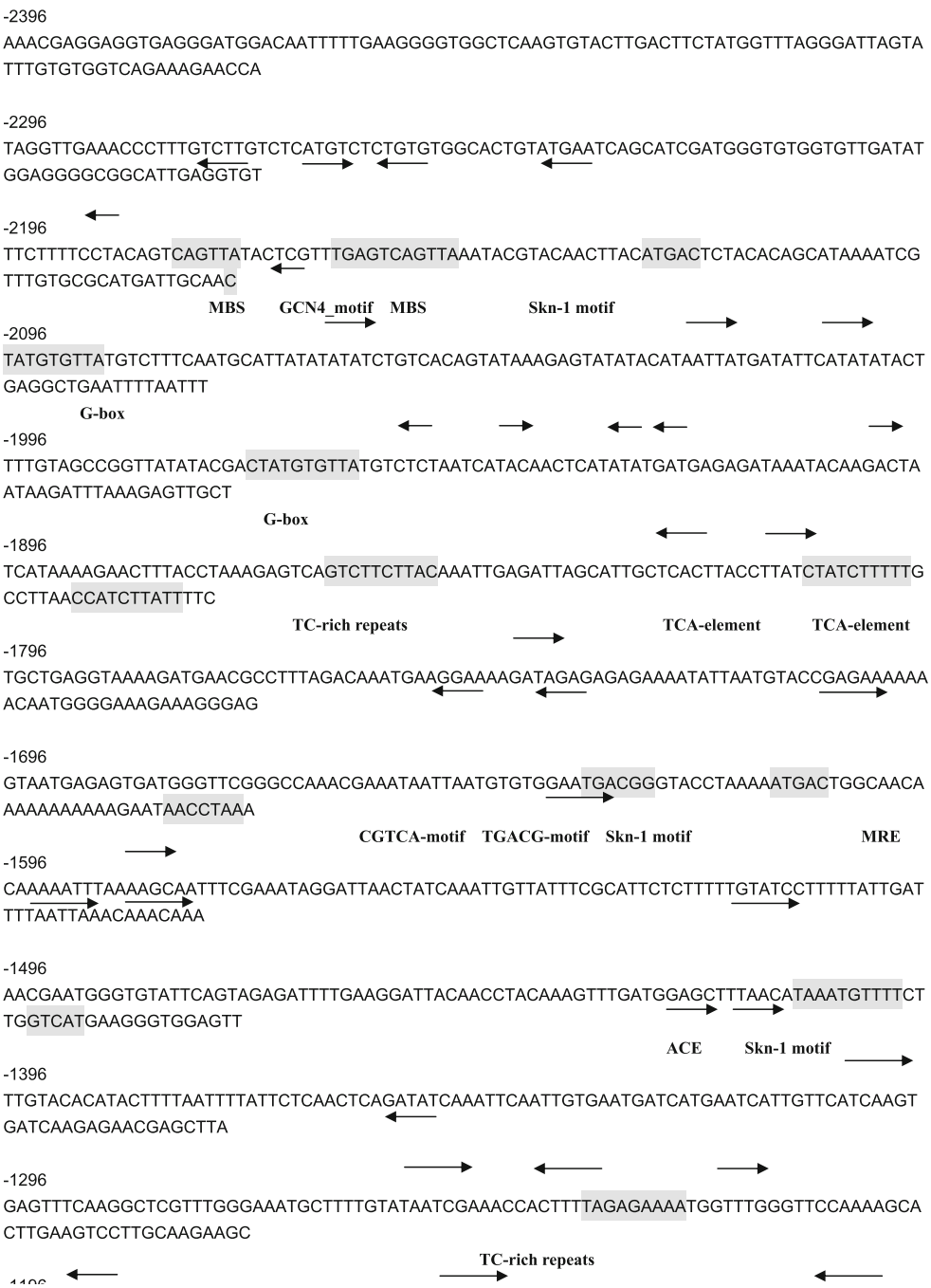

Fig. 4 S6PDH promoter nucleotide sequence from *M. domestica* "Gala". Translational start sites (+1) are shown in *box*. Motifs with significant similarity to previously identified *cis*-acting elements are *shaded*; names appear below elements. *Arrowheads* represent start directions of *cis*-regulatory elements

Table 2 Numbers and functions of *cis*-elements in *S6PDH* promoter sequences

Cis-element	Number	Function	Reference
ABRE	1	*Cis*-acting element involved in abscisic acid-responsiveness	Yamaguchi and Shinozaki 1993
ACE	2	*Cis*-acting element involved in light-responsiveness	Feldbrugge et al. 1994
ATC-motif	1	Part of conserved DNA modula involved in light-responsiveness	Liaud et al. 1990
ATCT-motif	1	Part of conserved DNA modula involved in light-responsiveness	Liaud et al. 1990
CGTCA-motif	2	*Cis*-acting regulatory element involved in MeJA-responsiveness	Rouster et al. 1997
Circadian clock	1	*Cis*-acting regulatory element involved in circadian control	Pichersky et al. 1985
GARE-motif	2	Gibberellin-responsive element	Pastuglia et al. 1997
G-box	4	*Cis*-acting regulatory element involved in light-responsiveness	An et al. 1993
GCN4-motif	1	*Cis*-regulatory element involved in endosperm expression	Kim and Wu 1990
LTR	1	*Cis*-acting element involved in low temperature-responsiveness	White et al. 1994
MBS	2	MYB-binding site involved in drought-inducibility	Yamaguchi and Shinozaki 1993
MRE	3	MYB-binding site involved in light-responsiveness	Feldbrugge et al. 1997
P-box	1	Gibberellin-responsive element	Kim and Wu 1992
Skn-1_motif	5	*Cis*-acting regulatory element required for endosperm expression	Takaiwa et al. 1991
TC-rich repeats	4	*Cis*-acting element involved in defense- and stress-responsiveness	Diaz de Leon et al. 1993
TCA-element	2	*Cis*-acting element involved in salicylic acid-responsiveness	Hennig et al. 1993
TGACG-motif	2	*Cis*-acting regulatory element involved in MeJA-responsiveness	Rouster et al. 1997

PlantCARE databases were used to identify several potential regulatory elements that corresponded to known *cis*-acting elements of eukaryotic genes. Detailed analysis of the *cis*-regulatory elements occurring in S6PDH promoters revealed that most elements were classified into three groups according to their functions: hormone-responsive elements, light-responsive elements, and stress-responsive elements (Table 2).

To test which part of the promoter sequence has high transcriptional activity, a series of deletions (-2396, -2122, -1719, -1066, -646, and -251) were transcriptionally fused to the promoterless reporter gene β-glucuronidase (GUS) (Fig.5). Each construct was introduced into tobacco leaves and tested for GUS activity. Here, the pC35SGUS construct was used as the positive control while the promoterless pC0390GUS served as the negative. The highest levels of GUS activity were detected in tobacco leaves harboring the construct containing the -1719 region of the S6PDH promoter. The -2122 and -1066 regions had a relatively low level of GUS activity. The lowest levels of GUS activity were detected in -2396, -646, and -251 regions (Fig.6). These results indicated that in the S6PDH promoter sequence, the -1719 region is more important for promoter activity than other regions.

Analysis of *cis*-regulatory elements of S6PDH promoters revealed that light-responsiveness motifs and stress-regulated motifs were present in the S6PDH promoter (Table.2). The effects of these elements were determined. We fused the 2396-bp fragment to the promoterless *GUS* reporter gene. This construct was introduced into tobacco leaves and tested for

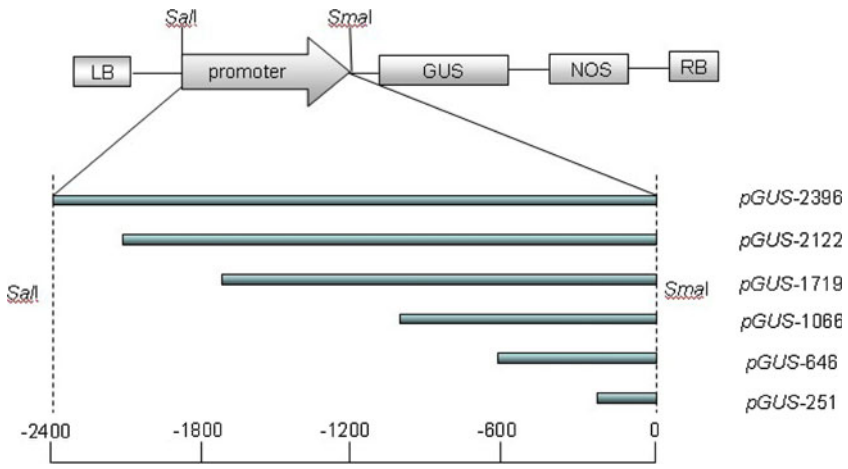

Fig. 5 Schematic representation of different deletion construction of S6PDH promoter

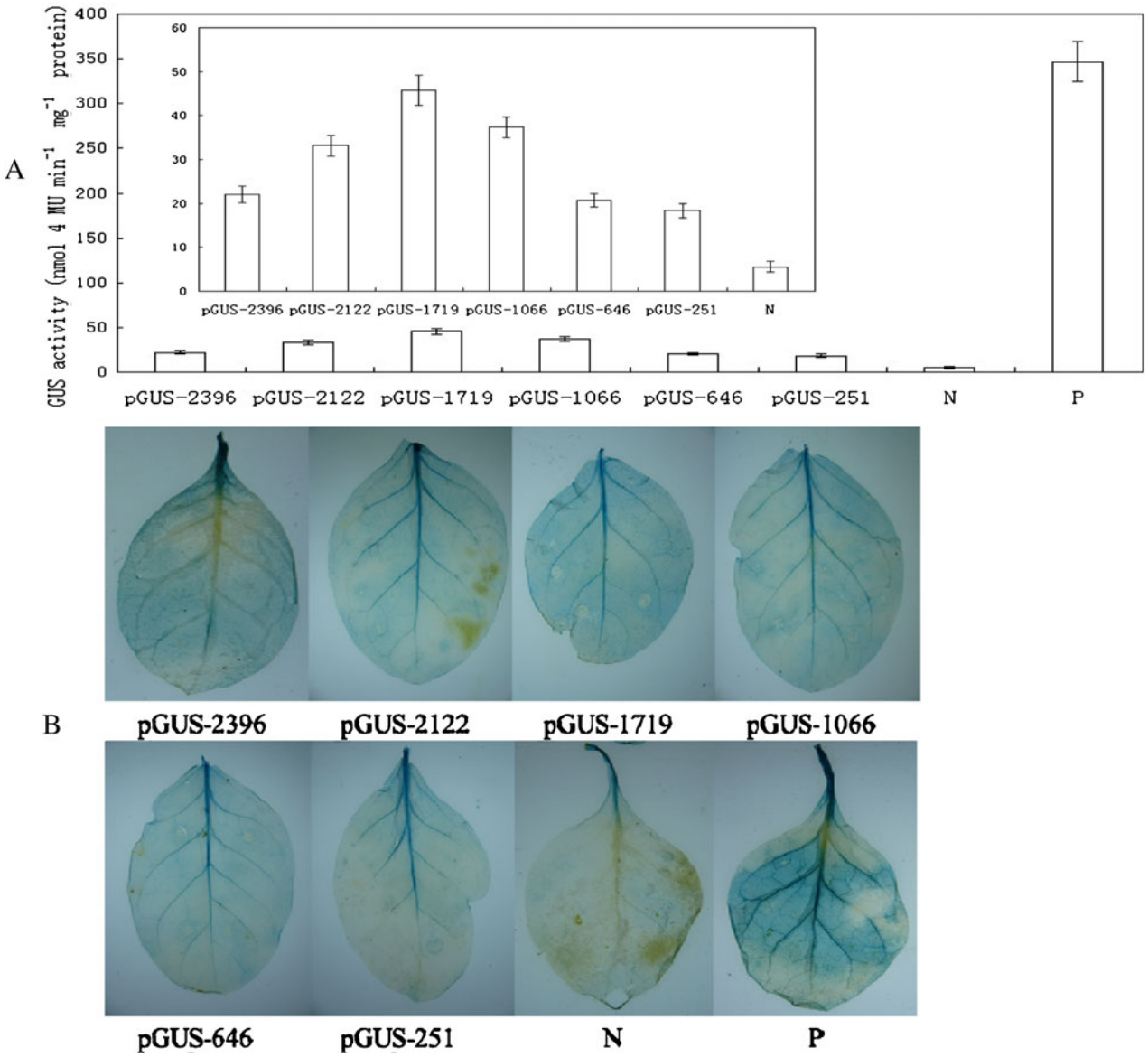

Fig. 6 GUS activity analysis of the deletions of S6PDH promoter in transiently transformed tobacco leaves. *N* represents negative control, *P* represents positive control. **a** GUS activity analysis. **b** Histochemical analysis

GUS activity. GUS assays displayed that S6PDH promoter was evidently induced by cold treatment, dark treatment, and ABA treatment. The inducible GUS activity after a 5-h cold treatment was approximately 2.21-fold, compared to the controls at 28°C (Fig.7a). S6PDH promoter-driven GUS activity in dark treatment was 6.61-fold lower than in control leaves (Fig.7b). With regard to ABA treatment, the S6PDH promoter was activated with a 2.76-fold higher inducible GUS activity compared with controls (Fig.7c).

Discussion

Although *S6PDH* has been studied in several plant species, little is known about its molecular characteristics. We systematically investigated its genomic structure, subcellular localization, and promoter.

In analyzing the molecular basis for *S6PDH* expression, we obtained two different genomic sequences — SgDNA1 and SgDNA2. The latter shares high sequence identity with apple S6PDH gDNA (AF057134). SgDNA1 and SgDNA2 differ in the sizes of their last three introns; however, they distribute in the same location on chromosome 10. It was reported that there are 16 S6PDH or S6PDH-like genes in the apple genome. They are located in chromosome 10 (Velasco et al. 2010). Therefore, SgDNA1 and SgDNA2 may be alleles. More work is needed to investigate whether there are different genomic sequence types of S6PDH in other *Malus* germplasm resource.

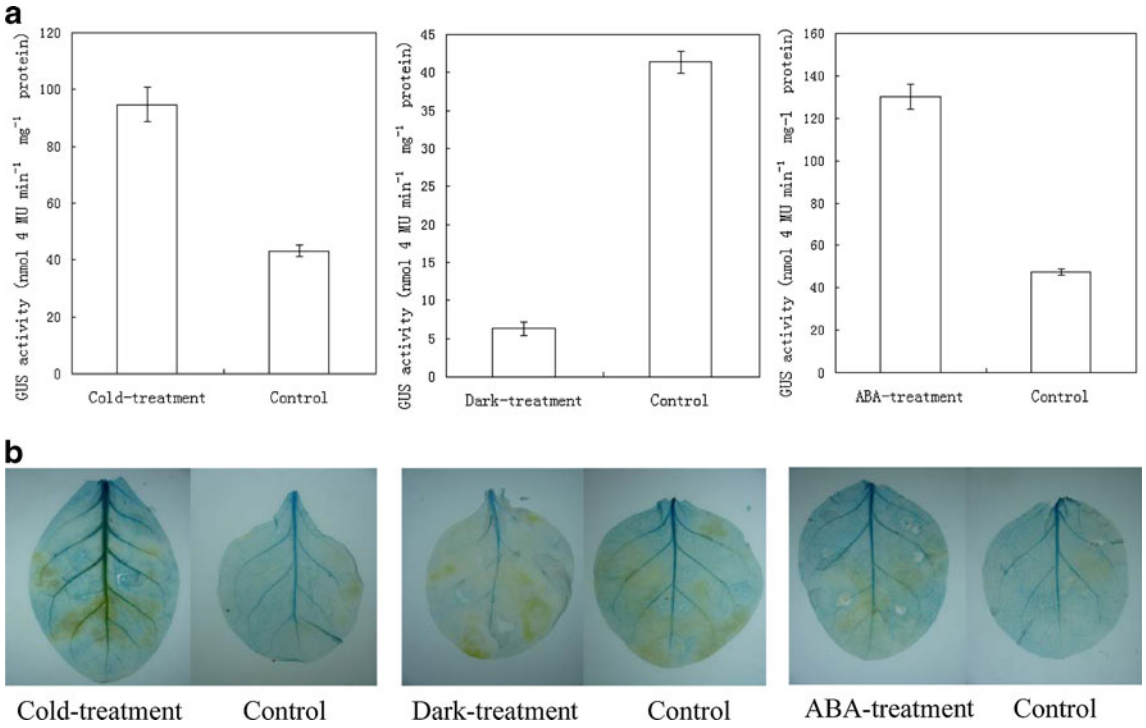

Fig. 7 S6PDH promoter response to different abiotic treatments. **a** GUS activity analysis, **b** Histochemical analysis (*left* cold treatment, *middle* dark treatment, *right* ABA treatment)

Little information was previously available about how S6PDH is distributed within the sub-cellular compartments of leaves. Our new discovery of its localization is essential for furthering our understanding on the mechanisms for sorbitol metabolism. Here, we have confirmed that S6PDH is distributed not only in the cytosol but also in the chloroplasts of leaf cells. Yamaki (1981) has also determined that S6PDH is found predominantly in the chloroplasts of apple cotyledons. Sorbitol and other sugars have also been detected in the vacuoles, protoplasts, and extracellular free spaces (Yamaki 1982). In mature peach leaves, sorbitol is present in the chloroplasts and cytosol, with the concentration being 1.5-fold higher in the former (Nadwodnik and Lohaus 2008). Wang et al. (2009) found that sorbitol dehydrogenase, the key enzyme for sorbitol metabolism, is distributed predominantly not only in the cytosol but also in the chloroplasts of the leaf. All of these results suggest that sorbitol can be synthesized and decomposed in the chloroplast and cytosol, which might explain our results for such localization on both sites in apple. In the cytosol, sorbitol that was synthesized by S6PDH will be used for phloem loading and export from source organs to sink organs. In the chloroplast, one possible explanation for S6PDH function is that S6PDH might be involved in regulating starch synthesis by Calvin cycle. On the other hand, sorbitol acts as an osmoregulatory substance to help maintain the osmotic balance. Therefore, the S6PDH present in the chloroplast and cytosol may take part in the regulation of the sorbitol concentration, maintaining the osmotic balance between chloroplast, cytosol, and apoplast.

To characterize the functional organization of the S6PDH promoter involved in expression, we made 5′-serially deleted promoter constructs fused to the GUS reporter gene and tested their expression by *Agrobacterium*-mediated transient assay system in tobacco leaves. The results demonstrated that the -1719 region of the S6PDH promoter had the highest levels of GUS activity. One possible explanation is that both activation and repression regulatory elements co-exist in S6PDH promoter sequence. Positive regulatory elements, maybe, are present at positions from -1719 to -251, and negative regulatory elements may be located from -2396 to -1719. Examining the *S6PDH* promoter sequence, we found several stress-regulated and light-responsive elements, such as ABRE, CGTCA-motif, LTR, MBS, TC-rich repeats, TGACG-motif, ACE, ATC-motif, ATCT-motif, G-box, and MRE. These are homologous to *cis*-acting elements previously identified in plants. This paper displayed that S6PDH promoter was evidently induced by stress and light treatment.

Many physiological processes in plants are affected by different stresses (Gao et al. 2010; Stolf-Moreira et al. 2011; Zhang et al. 2011). Sorbitol is induced to accumulate in abiotic stress and has been proposed to act as an osmoregulatory substance to help maintain the osmotic balance under adverse growing conditions. As the key enzyme in sorbitol biosynthesis, expression of *S6PDH* can also be enhanced by ABA

treatment or the exposure to low temperatures and high salinity in apple, as well as sorbitol content (Kanayama et al. 2006). A linear increase in S6PDH activity with drought stress correlates with a significant accumulation of sorbitol in phloem of peach (Escobar-Gutiérrez et al. 1998). These results showed that S6PDH gene and S6PDH promoter had similar expression pattern against stress conditions. It means that the transcriptional level regulation may be a key step for S6PDH gene expression at adverse situation.

In summary, two different genomic sequences for *S6PDH* from apple, designated as SgDNA1 and SgDNA2, were isolated and may be alleles on chromosome 10. Our immuno-electron microscopy revealed that S6PDH is localized on the cytosol and chloroplasts of apple leaves. Analysis of its gene promoter region leads us to believe that this promoter responds to light and various environmental stresses. Therefore, we can conclude that *S6PDH* is multifunctional for sorbitol biosynthesis under various stresses and photosynthate demands.

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