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Molecular evidence of sorbitol dehydrogenase in tomato, a non-Rosaceae plant

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Abstract

The enzyme NAD-dependent sorbitol dehydrogenase (SDH) is well characterized in the Rosaceae family of fruit trees, which synthesizes sorbitol as a translocatable photosynthate. Expressed sequence tags of *SDH*-like sequences have also been generated from various non-Rosaceae species that do not synthesize sorbitol as a primary photosynthetic product, but the physiological roles of the encoded proteins in non-Rosaceae plants are unknown. Therefore, we isolated an *SDH*-like cDNA (SDL) from tomato (*Lycopersicon esculentum* Mill.). Genomic Southern blot analysis suggested that *SDL* exists in the tomato genome as a single-copy gene. Northern blot analysis showed that SDL is ubiquitously expressed in tomato plants.

Recombinant SDL protein was produced and purified for enzymatic characterization. SDL catalyzed the interconversion of sorbitol and fructose with NAD (H). SDL showed highest activity for sorbitol among the several substrates tested. SDL showed no activity with $NADP^+$. Thus, SDL was identified as a SDH, although the K_m values and substrate specificity of SDL were significantly different from those of SDH purified from the Japanese pear (*Pyrus pyrifolia*), a Rosaceae fruit tree. In addition, tomato was transformed with antisense SDL to evaluate the contribution of SDL to SDH activity in tomato. The transformation decreased SDH activity to approximately 50% on average. Taken together, these results provide molecular evidence of SDH in tomato, and SDL was renamed LeSDH.

Keywords: *Lycopersicon esculentum* Mill.; Solanaceae; Tomato; cDNA cloning; Recombinant protein expression; Antisense transformation; Sorbitol dehydrogenase; Carbohydrate; Sorbitol; Fructose

1. Introduction

Sorbitol is important in translocating photosynthate in fruit trees of the Rosaceae family (Kanayama et al., 1992; Kanayama, 1998; Sakanishi et al., 1998). NAD-dependent sorbitol dehydrogenase (SDH) catalyzes the oxidation of sorbitol to fructose. SDH has been purified from Japanese pear fruit (Oura et al., 2000). The expression analysis of *SDH* cloned from apple cDNA demonstrated the importance of SDH in the metabolism of sorbitol that is translocated to fruit (Yamada et al., 1998, 1999; Park et al., 2002).

SDH has also been found in plants that are not in the Rosaceae and that synthesize sucrose for translocation of photosynthate. SDH activity was detected in a crude extract from germinating soybean seeds (Kuo et al., 1990), and SDH was partially purified from developing maize endosperm (Doehlert, 1987) and from the shoot axes of *Viscum album*, a parasitic plant (Wanek and Richter, 1993).

Recently, plant genome mapping projects have revealed that SDH-like sequences are widespread in the plant kingdom, and are present in the expressed sequence tag (EST) databases of several plant species (Fig. 1). Nevertheless,

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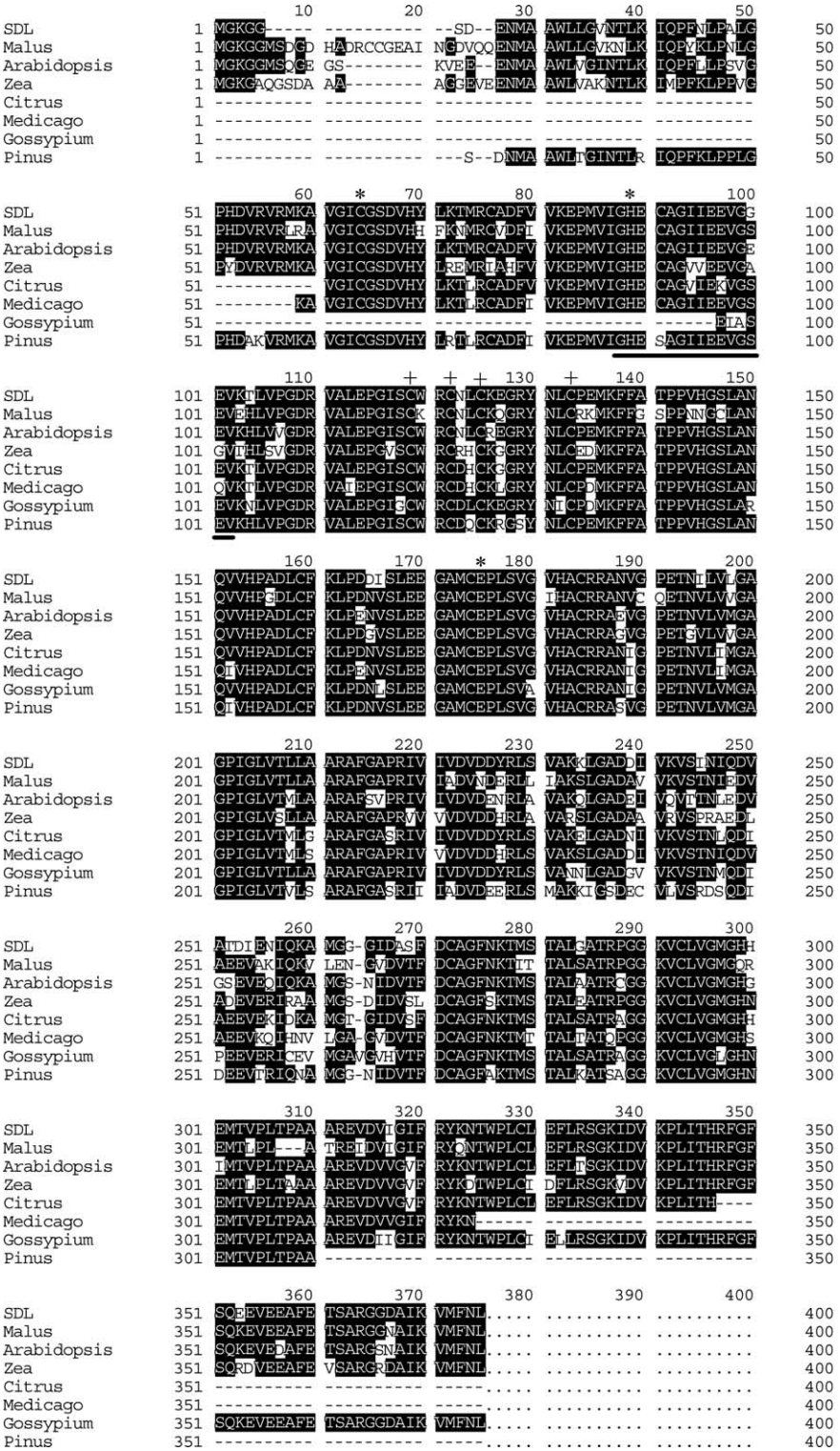

Fig. 1. Multiple alignment of the deduced tomato SDL amino acid sequence, the apple SDH sequence, and the SDH-like sequences in EST from species of different plant families. Black shading indicates identical amino acids. Asterisks and plus signs indicate conserved amino acid residues in the catalytic zinc binding site and in the structural zinc binding site, respectively. The zinc-containing alcohol dehydrogenase signature is underlined. SDL (accession number AB183015); *Malus domestica*, apple SDH (AB016256); *Arabidopsis thaliana* (AF370161); *Zea mays* (BT016754); *Citrus paradisi* × *Poncirus trifoliata* (CX668813); *Medicago sativa* (CB894631); *Gossypium raimondii* (CO082515); *Pinus taeda* (CO361351).

little information exists about the physiological roles of the proteins encoded by these genes. The ultimate goal of this research was to understand the significance of the wide-spread *SDH*-like genes. As a first step, we now provide molecular evidence of *SDH* genes in non-Rosaceae plants. This study is a molecular and biochemical characterization

of an *SDH* homolog in tomato, a species that utilizes sucrose to translocate photosynthate.

2. Results and discussion

2.1. Deduced amino acid sequence of SDL, a tomato SDH homologue

A tomato cDNA fragment was cloned by the rapid amplification of cDNA ends (RACE), based on an EST clone sequence that was homologous to apple SDH. This cloned fragment, representing an *SDH* homologue in tomato, contained the entire coding sequence and was defined as *SDL*. The SDL amino acid sequence from tomato was compared with the sequence from apple SDH and SDH-like sequences from several other species (Fig. 1). Apple SDH has been characterized using its recombinant protein (Yamada et al., 1998), but other SDH-like sequences have not been characterized molecularly or biochemically. Full-length cDNA sequences from *Arabidopsis thaliana* and *Zea mays* are available in DNA Data Bank of Japan (DDBJ) as are partial sequences from *Citrus paradisi* × *Poncirus trifoliata, Medicago sativa, Gossypium raimondii*, and *Pinus taeda*. The tomato SDL protein consists of 355 amino acids.

The amino-terminal region is slightly shorter in SDL from tomato than in comparable sequences from apple, *A. thaliana*, and *Z. mays*. The tomato SDL amino acid sequence is 76% identical with apple, 84% with *A. thaliana*, and 77% with *Z. mays* sequences. The corresponding sequences of tomato SDL are 88% identical with the partial sequence from *C. parodisi* × *Poncirus trifoliata*, 84% identical with that from *M. sativa*, 82% identical with that from *G. raimondii*, and 80% identical with that from *P. taeda*. The closest Blast hit was the sequence of *A. thaliana* among full-length cDNA sequences and was that of *C. paradisi* × *Poncirus trifoliate* among partial sequences. The SDL sequences include a zinc-containing alcohol dehydrogenase signature, a catalytic zinc binding site, and a structural zinc binding site that are conserved in SDH (Yamada et al., 2001).

2.2. Southern and Northern blot analyses

Genomic DNA extracted from tomato leaves was cut with *Eco*RI, *Hin*dIII or *Xba*I, and Southern hybridization was performed using the tomato *SDL* cDNA as a probe (Fig. 2). The *Eco*RI and *Hin*dIII digestions resulted in a single band, while the *Xba*I digestion showed a strong band along with a weak band. These results suggest that *SDL* is a single-copy gene in the tomato genome.

SDL mRNA was detected in all the tissues tested, as shown in Fig. 3A. An additional, slightly smaller, band was observed in some lanes. Park et al. (2002) characterized four cDNAs (*MdSDH1-4*) encoding the isoforms of apple SDH. They showed that the expression of *MdSDH2, 3*, and 4 is restricted to sink tissues, including young leaves, stems, roots, and maturing fruits. However, we found that the expression of *SDL* was not restricted to sink tissues. Among the organs we tested, the level of *SDL* mRNA was highest in flowers (Fig. 3A). We examined *SDL* expression in individual floral organs in detail (Fig. 3B) and found that *SDL* was expressed similarly in all floral organs.

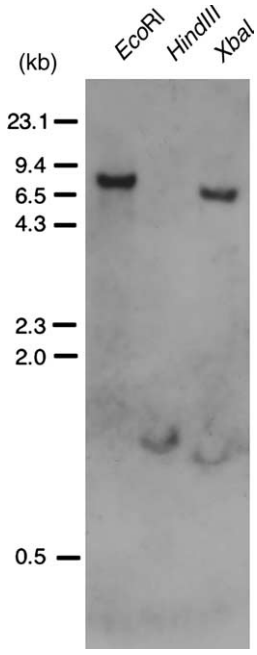

Fig. 2. Genomic Southern blot analysis of tomato *SDL*. Genomic DNA was digested with the indicated restriction enzymes. The blot was probed with the *SDL* EST fragment.

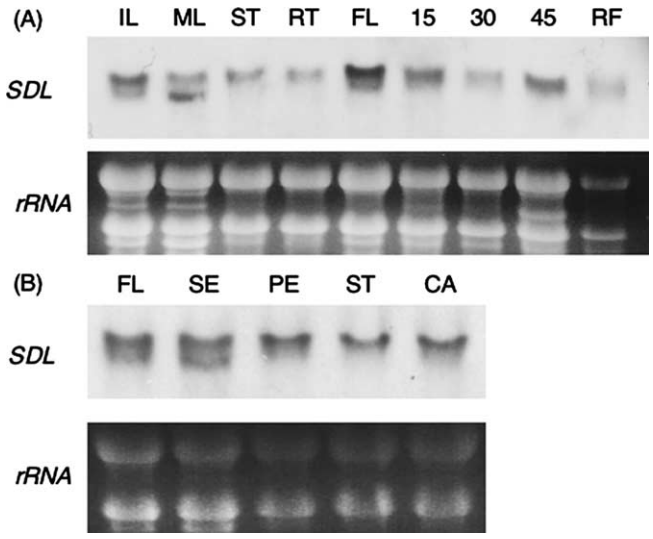

Fig. 3. Northern blot analysis of tomato *SDL* mRNA. (A) Each lane was loaded with 10 μg of total RNA isolated from immature leaves (IL), mature leaves (ML), stems (ST), roots (RT), flowers (FL), fruit at 15, 30, and 45 days after flowering (15, 30, and 45), and ripe fruit (RF). (B) Each lane was loaded with 10 μg of total RNA isolated from individual floral organs: whole flowers (FL), sepals (SE), petals (PE), stamens (ST), and carpels (CA). The blots were probed with the *SDL* EST fragment. rRNA stained with ethidium bromide was used as a control for loading.

Collectively, the expression of *SDL* in tomato plants was ubiquitous.

2.3. Analysis of recombinant SDL protein

The SDS–PAGE analysis of the fractions collected during the preparation of recombinant SDL protein is shown in Fig. 4. The induced glutathione-S-transferase (GST) fusion protein was detected in an insoluble fraction of *E. coli*, and the fusion protein was solubilized by urea. After the urea treatment, zinc ions were essential for maintaining the activity of the recombinant SDL during dialysis. This confirmed that SDL contained zinc binding sites, as described in its amino acid sequence (Fig. 1). The solubilized fusion protein was completely cleaved by protease and was purified using glutathione–Sepharose 4B. Finally, the recombinant SDL was purified to homogeneity, although a weak band for GST was observed on SDS–PAGE. The purified recombinant SDL was used for the following experiments.

The products of the recombinant SDL reactions were first examined by gas chromatography (Fig. 5). The results indicated that SDL catalyzes the interconversion of sorbitol and fructose using NAD(H). The substrate and coenzyme specificity are shown in Table 1. SDL showed the highest activity for sorbitol with NAD^+ and no activity with $NADP^+$. This result indicated that SDL is NAD-dependent sorbitol dehydrogenase. Although SDL showed comparatively high activity for iditol and ribitol, it did not show activity for *myo*-inositol, glycerol, and ethanol, which are common in plants. Several differences were found in the substrate specificity of SDL and SDH purified from Japanese pear fruit (Oura et al., 2000). The relative activity of SDL was higher for ribitol, meso-erythritol, and L-arabitol than that of the pear SDH (14% for ribitol, 1% for meso-erythritol, 2% for L-arabitol), but was lower for xylitol than that of the pear SDH (77% for xylitol).

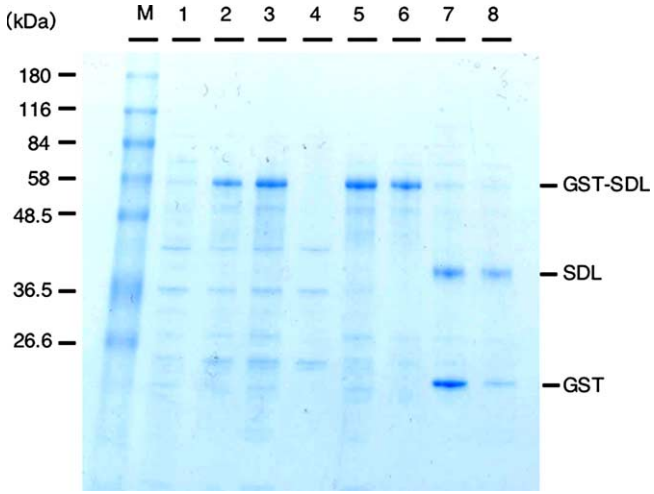

Fig. 4. Preparation of recombinant SDL monitored by SDS–polyacrylamide gel electrophoresis. Molecular weight markers (M); crude extract from *E. coli* before (1) and after (2) the induction of GST–SDL fusion protein expression; total fraction (3), soluble fraction (4), and insoluble fraction (5) after the lysis of *E. coli*, GST–SDL fusion protein solubilized from the insoluble fraction by urea (6); GST and SDL cleaved by protease (7); purified SDL after removal of GST (8). The protein equivalent of approximately 1% of each fraction was electrophoresed and stained with Coomassie Brilliant Blue.

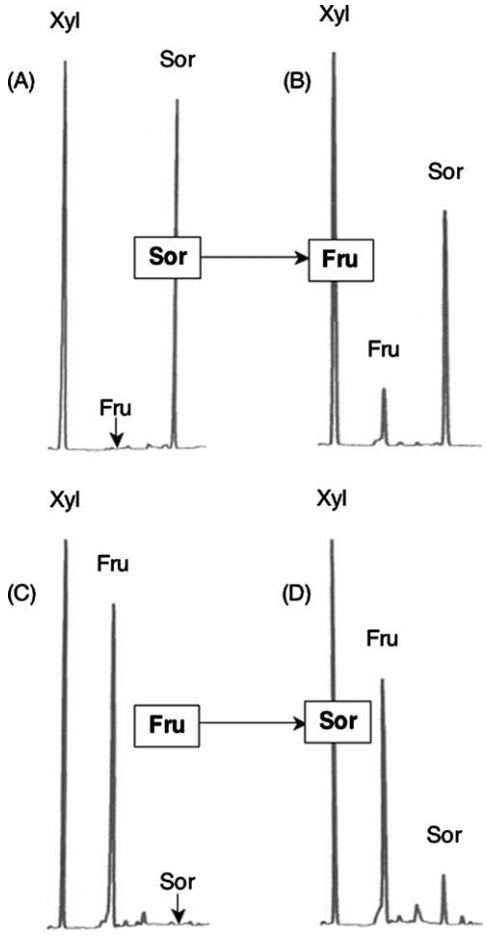

Fig. 5. Gas chromatographic analysis of the products of the SDL reactions. Chromatograms before (A,C) and after (B,D) the reactions are shown. Sorbitol (A,B) or fructose (C,D) was used as a substrate. Xylitol was added before the trimethylsilylation of the products, as an internal standard for gas chromatography.

The enzymatic properties of SDL were compared with those of Japanese pear SDH (Oura et al., 2000) and ketose reductase (SDH) partially purified from the endosperm of maize (Doehlert, 1987) (Table 2). The K_m values of the SDHs from the three species were significantly lower for sorbitol than for fructose, showing their high affinity for sorbitol. In a comparison of the three SDHs, the K_m values of SDL for both substrates were more similar to those of maize SDH than those of Japanese pear SDH; that is, the K_m values of Japanese pear SDH were more than 10-fold those of SDL and maize SDH for both substrates. The V_{max} values of the SDHs from the three species were higher for fructose reduction than for sorbitol oxidation. The effects of pH on the activity were also similar among the SDHs: the greatest activity was observed at alkaline pH for sorbitol oxidation and at about neutral pH for fructose reduction.

Table 1
Substrate and coenzyme specificity of SDL[a]

Substrate	Relative activity (%)
D-Sorbitol	100
L-Iditol	79
Ribitol	60
Xylitol	29
Erythritol	13
L-Arabitol	13
D-Mannitol	6
myo-Inositol	n.d.[c]
Glycerol	n.d.
Ethanol	n.d.
NADP[b]	n.d.

[a] Enzyme activity was assayed in 400 mM of each substrate with 1 mM NAD^+.
[b] Activity was assayed in 400 mM sorbitol with 1 mM $NADP^+$.
[c] n.d., not detected.

The tomato SDL was identified as SDH using the purified recombinant protein. However, when the enzymatic properties of SDL were compared with those of Japanese pear SDH purified to homogeneity, some properties differed significantly between the two.

2.4. Antisense suppression of SDL expression

To our knowledge, transformation with the SDH gene has not been reported for any plant. We transformed tomato with antisense *SDL* to demonstrate the contribution of *SDL* to SDH activity in tomato. The SDH activity was significantly lower in antisense plants than in control plants (Table 3). The lowest activity in the antisense line was approximately 10% that of the control line. The result showed that *SDL* is a major gene for SDH activity in tomato.

Roessner-Tunali et al. (2003) and Cataldi et al. (1998) detected a low amount of sorbitol in tomato. Furthermore, sorbitol-6-phosphate dehydrogenase, which is a key enzyme in sorbitol biosynthesis (Kanayama et al., 1992), was detected in tomato by immunoblot analysis (Mehta et al., 1991); we also identified sorbitol-6-phosphate dehydrogenase activity in tomato (data not shown). These facts suggest the presence of sorbitol in tomato, and thus SDL might play a role in sorbitol metabolism. On the other hand, arabitol, which can be a substrate of SDL (Table 1), accumulates in the leaves of tomato plants infected with fungal pathogens (Clark et al., 2003). This accumulation is important for fungal pathogenicity; therefore, SDL might have antifungal activity by metabolizing arabitol. The physiological function of SDL will be clarified by investigating the transgenic tomato plants with antisense *SDL*.

Table 3
SDH activity in *SDL* antisense plants

Line	Number of plants assayed	SDH activity[a] (μmol min^{-1} mg^{-1} protein)	% of control
Control	4	0.185(0.014)	
Antisense	9	0.091 (0.013)	49.2

[a] Each value represents the mean (the associated standard error).

2.5. Concluding remarks

The results of this study provide molecular evidence for the presence of an SDH in tomato, and thus *SDL* was renamed *LeSDH* (accession number AB183015). Information on sorbitol metabolism in non-Rosaceae plants is scarce at the molecular level because of the minute quantity of sorbitol present. However, this small quantity of sugar could be important in plant growth, as was recently shown for trehalose (Schluepmann et al., 2003). For example, small quantities of sorbitol are associated with the transport of boron, which is essential for growth of vascular plants (Bellaloui et al., 1999). mRNAs for SDH-like proteins with amino acid sequences that are 80% or more identical to that of SDL are expressed in non-Rosaceae plant families, including monocot and gymnosperm families (Fig. 1). To our knowledge, this is the first investigation to use recombinant protein and antisense transformation to show molecular evidence for sorbitol dehydrogenase, which is widespread in the plant kingdom.

3. Experimental

3.1. Plant materials

Lycopersicon esculentum Mill. cv. Momotaro was used for the molecular cloning of *SDL*, Southern and Northern blot analyses, and the production of recombinant protein. *Lycopersicon esculentum* Mill. cv. Alisa Craig was used for the antisense transformation.

Table 2
Kinetic characteristics and pH optima of SDL, Japanese pear SDH, and maize ketose reductase (SDH)

Plant	Substrate	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1} protein)	pH optimum	Reference
Tomato	Sorbitol	2.39	0.378	10.5	This study
	Fructose	99.5	2.13	7.5	
Japanese pear	Sorbitol	96.4	64.8	9.0	Oura et al. (2000)
	Fructose	4239	162.6	7.0	
Maize	Sorbitol	8.45	5.87	9.0	Doehlert (1987)
	Fructose	136	21.2	6.0	

3.2. Cloning of cDNA encoding SDL

The total RNA was extracted from mature tomato leaves by the SDS–phenol method (Kanayama et al., 1997). The SDH-like EST sequence (accession number BI203186) was amplified using an RNA PCR kit (AMV) Ver. 2.1 (Takara) and was cloned into pT7Blue vector (Novagen). Next, 5′-RACE was carried out with poly $(A)^+$ RNA prepared using Oligotex-dT30 (Takara), and the cDNA that contained the open reading frame encoding SDL was cloned into pT7Blue vector. A high-fidelity enzyme was used for the 5′-RACE. The BigDye Terminator v3.0 Cycle Sequencing Ready Reaction kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) were used for sequencing.

3.3. Southern and Northern blot analyses

The genomic DNA extraction from tomato leaves and the Southern blot analysis were based on the method of Kanayama et al. (1997). The EST clone for *SDL* was labeled using the PCR DIG probe synthesis kit (Roche) for use as a probe. After hybridization, the membrane was washed in 0.2× SSC at 65 °C. The SDS–phenol method (Kanayama et al., 1997) was used to extract total RNA from various tomato plant organs for Northern blot analyses, which employed the method of Odanaka et al. (2002). The EST clone for *SDL* was labeled using the PCR DIG probe synthesis kit (Roche) for use as a probe.

3.4. Production of recombinant SDL protein

The open reading frame of *SDL* was cloned into the pGEX-6P-3 expression vector (Amersham Bioscience) to make pGEX-SDL. *E. coli* BL21 transformed with pGEX-SDL was cultured to an OD_{600} of 0.5 in Luria–Bertani medium supplemented with ampicillin at 37 °C, 0.2 mM isopropyl β-D-thiogalactopyranoside was added, and the culture was continued for 3 h more at 37 °C. The *E. coli* was collected by centrifugation and lyzed using BugBuster HT (Novagen). The GST–SDL fusion protein was found in the pellet after centrifugation; the fusion protein was extracted in 100 mM Tris–HCl (pH 8.0) containing 6 M urea, 1 mM EDTA, and 1 mM DTT for 1 h at 4 °C. After centrifugation, the supernatant was dialyzed against 100 mM Tris–HCl (pH 8.0) containing 2 M urea, 1 mM EDTA, 1 mM DTT, and 1 mM $ZnSO_4$ for 1 h at 4 °C and then against 100 mM Tris–HCl (pH 8.0) containing 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM $ZnSO_4$ for 16 h at 4 °C. After dialysis and centrifugation, the protein in the supernatant was bound to glutathione–Sepharose 4B (Amersham Bioscience) and the GST was removed from the recombinant SDL protein using PreScissin protease (Amersham Bioscience). The expression and purity of the recombinant protein were checked by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970).

3.5. Enzyme assay using recombinant SDL protein

The products of the SDL reaction were examined by gas chromatography. The reaction mixture consisted of 50 mM Tris–HCl (pH 9.0), 10 mM sorbitol, and 20 mM NAD^+ or of 50 mM Tris–HCl (pH 7.5), 10 mM fructose, and 40 mM NADH. After 4 h of incubation at 37 °C, the reaction was halted by boiling. Xylitol was added as an internal standard, and after trimethylsilylation, the reaction mixture was analyzed on a gas chromatograph G-300 (Hitachi, Tokyo, Japan).

The substrate specificity of SDL was examined in 50 mM Tris–HCl (pH 9.5) containing 1 mM NAD^+ and 400 mM substrate, as described in Table 1. The coenzyme specificity of SDL was examined in 50 mM Tris–HCl (pH 9.5) containing 1 mM $NADP^+$ and 400 mM sorbitol (**1b**). For the determination of the K_m and V_{max} values, the reaction mixture for sorbitol oxidation consisted of 50 mM glycine–NaOH (pH 10.5), 1 mM NAD^+, and various concentrations of sorbitol. For fructose reduction, the reaction mixture consisted of 50 mM Tris–HCl (pH 7.5), 0.1 mM NADH, and various concentrations of fructose. These reactions were carried out at 37 °C.

The effect of pH on the SDL reaction was examined in 50 mM Tris–HCl (pH 8.0–9.5) and 50 mM glycine–NaOH (pH 9.0–11.0) for sorbitol oxidation, or in 50 mM Tris–acetate (pH 5.5–7.5) and 50 mM Tris–HCl (pH 7.0–9.0) for fructose reduction. Each reaction was carried out in a buffer that contained 400 mM substrate and 1 mM NAD^+ or 0.1 mM NADH at 37 °C.

3.6. Transformation of tomato with antisense LeSDH

The β-glucuronidase gene in the binary vector pBI121 was replaced with the insert of the *SDL* EST clone in an antisense direction. This antisense *SDL* vector with the cauliflower mosaic virus 35*S* promoter was introduced into *Agrobacterium tumefaciens* LBA4404. The transformation was carried out using tomato cotyledons as described by Odanaka et al. (2002). After shoot and root formation, the independent transformants were acclimated and checked by PCR for the transgene (Fig. 6). Primers used

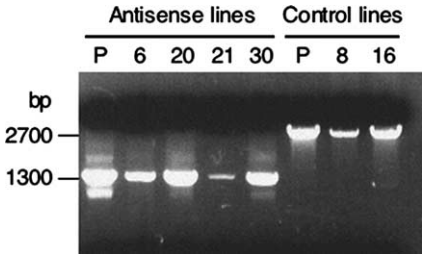

Fig. 6. PCR analysis for the transgene in *SDL* antisense plants. One example is shown. PCR bands in *SDL* antisense lines are shorter than those in control lines since the antisense insert, which is approximately 500 bp EST clone of *SDL*, is shorter than the insert of β-glucuronidase gene, which is approximately 1900 bp, in the control vector, pBI121. Each vector for the transformation was used as a positive control (P) for the PCR analysis.

for the PCR were 5′-CAAACCAAGGCAAGTAATAG-3′ in 35*S* promoter and 5′-CTATATTTTGTTTTCTATCG-CG-3′ in *nos* terminator. When the first flower opened, the third and fourth leaves below the inflorescence were sampled for enzyme assays. The method described by Suzuki et al. (2001) was used to extract the crude enzyme and to determine SDH activity.

3.7. Protein determination

The protein determination was carried out by the method of Bradford (1976) using bovine serum albumin as a standard.

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