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Intercellular Localization of Acid Invertase in Tomato Fruit and Molecular Cloning of a cDNA for the Enzyme

Takahide Sato[1,3], Tomoyoshi Iwatsubo[1], Michiko Takahashi[1], Hiroki Nakagawa[1], Nagao Ogura[1] and Hitoshi Mori[2]

[1] *Division of Bioproduction Science, Faculty of Horticulture, Chiba University, 648 Matsudo, Chiba, 271 Japan*
[2] *Division of Biological Regulation, National Institute for Basic Biology, Okazaki, 444 Japan*

The localization of acid invertase (AI, EC 3.2.1.26) in tomato fruits was studied. AI was localized in the intercellular fraction (cell wall fraction). A cDNA encoding a wall-bound form of AI from tomato fruits was cloned and its nucleotide sequence was determined. The cloned cDNA was 2363 base pairs long and contained an open reading frame of 1908 base pairs which encoded a polypeptide of 636 amino acids. RNA blot analysis indicated that the mRNA for the acid invertase was about 2.5 kb in length. The levels of the mRNA were low at the mature green stage but increased during ripening of fruit.

Key words: Acid invertase — cDNA cloning — Ripening — Tomato fruit (*Lycopersicon esculentum* M.).

The activity of acid invertase (EC 3.2.1.26) in tomato fruits is low at the mature green stage but increases during the ripening of fruits (Nakagawa et al. 1970, Endo et al. 1990). AI activity in tomato fruits has been shown to be inversely related to the sucrose content of fruits (Miron and Schaffer 1991, Yelle et al. 1991). Most of the AI activity in mature tomato fruits can be extracted from insoluble cell debris with a buffer of high ionic strength and, therefore, AI has been classified as a wall-bound enzyme or, possibly, an extracellular enzyme. However, since sucrose is believed to be present in vacuoles, the possible role of extracellular invertase in relation to the level of vacuolar sucrose should be reevaluated. In fact, Yelle et al. (1991) reported that the distribution of AI in soluble and wall-bound fractions varied with the pH of the extraction buffer. Therefore, it is necessary to obtain more reliable information on the localization of AI in tomato fruits. Examination of intercellular washing fluid has been successfully employed in efforts to determine the localization of other enzymes (Ishige et al. 1991). If AI of tomato fruits is present in the cell wall region, the enzyme should be detectable in the intercellular washing fluid.

We reported previously that the increase in AI activity during ripening of tomato fruits was due to synthesis of the enzyme de novo (Endo et al. 1990) and that the levels of translatable mRNA, as measured with an in vitro translation system, increased during ripening. However, it has not been conclusively demonstrated that the increase in levels of the mRNA is regulated at the transcriptional level. Such increases can only be demonstrated by cloning of the cDNA for AI and subsequent Northern blot analysis of gene expression.

In this study, we first examined the localization of AI in tomato fruits, then we cloned a cDNA for the enzyme and, finally, we measured the changes in the level of the mRNA during ripening of fruit.

Materials and Methods

Plant material—Fruits of tomato (*Lycopersicon esculentum* M. cv. Super First) were grown on the farm of the Japan Horticultural Productivity Institute, Matsudo, Chiba. Fruits were harvested at the designated stages as described in the text.

Abbreviations: AI, acid invertase; CW, cell wall-bound; CTAB, cetyl triethylammonium bromide ; IWF, intercellular washing fluid; 2-ME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; S, soluble.

[3] To whom correspondence should be addressed.

Determination of partial amino-acid sequences of purified AI—AI of tomato fruits was purified previously (Endo et al. 1990) and has been stored at −80°C. The preparation of purified protein was fractionated by SDS-PAGE on a 10% polyacrylamide gel (Laemmli 1970) and transferred to a polyvinylidene difluoride membrane (Immobilon,; Millipore, MA, U.S.A.). The membrane was stained with Coomassie Brilliant Blue, and two polypeptides of about 50 and 20 kDa were found. The portions of the membrane with the two polypeptides were excised and their N-terminal amino acid sequences were determined with a gas-phase microsequencer (Matsudaira 1987). Oligonucleotide mixtures of sense (primer 50) and antisense (primer 20) sequences were synthesized that corresponded to two six-amino-acid sequences selected from the N-terminal amino acid sequences of the 50-kDa and 20-kDa polypeptides, respectively (Fig. 1).

Extraction of RNA and construction of a cDNA library—Nucleic acids were extracted from ripe tomato fruits by the SDS-phenol method as described previously (Sato et al. 1978). The resultant crude fraction, which contained soluble polysaccharides, was dissolved in 0.7 M NaCl in buffer A (50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 1% CTAB), and an equal volume of buffer A was added. After 1 h, the precipitate of nucleic acids was collected by centrifugation at 3,000 × *g* for 10 min and washed with ethanol. The nucleic acids were dissolved in 2 M LiCl and placed on ice at least for 2 h. The resultant precipitate was collected by centrifugation at 10,000 × *g* for 20 min at 0°C. After the precipitate had been washed with ethanol, it was dissolved in distilled water and used as total RNA. Poly(A)$^+$-RNA was isolated from total RNA by column chromatography on oligo(dT)-cellulose (Aviv and Leder 1972). The first strand of cDNA for tomato poly(A)$^+$-RNA was synthesized by using primer 20, and the single-stranded cDNA was used as the template for amplification of a cDNA fragment between primer 50 and 20 by PCR (Arai et al. 1992). cDNA produced by PCR was purified by agarose gel electrophoresis, and a labeled probe was prepared from the cDNA by use of a Random Primer DNA Labeling Kit (Takara Shuzo, Kyoto, Japan). A cDNA library for poly(A)$^+$-RNA from ripe tomato fruits was constructed in Bluescript SK(−) (Stratagene, CA, U.S.A.) by the vector-primer method, as described previously (Mori et al. 1991). About 2×10^5 colonies of transformed *E. coli* DH 5*α* were screened with the ^{32}P-labeled probe. The nucleotide sequence of the DNA was determined by the dideoxynucleotide chain-termination method (Sanger et al. 1977).

Analysis of RNA—Total (10 μg per lane) or poly(A)$^+$-RNA (2 μg per lane) was fractionated on agarose gels that contained formaldehyde (Sambrook et al. 1989). RNA was transferred to nylon membranes (Hybond-N$^+$, Amersham Corp., Buckinghamshire, England). Prehybridization was performed in a solution of 50% formamide, 6 × SSPE, 0.5% SDS, 5% Irish Cream (R. and A. Bailey & Co. Dublin, Ireland), and 20 μg ml^{-1} denatured salmon sperm DNA at 42°C for 1 h. Hybridization was carried out in the same solution with the ^{32}P-labeled probe at 42°C for 18 h. The blots were washed twice with 3 × SSC and 0.1% SDS at 60°C for 30 min.

General methods—General methods for manipulation of DNA and RNA were those described by Sambrook et al. (1989).

Preparation of intercellular washing fluid—Five g of tissue slices (about 5 mm in thickness) were vacuum-infiltrated for 5 min with 10 ml of buffer B (20 mM potassium phosphate, pH 5.5, 0.5 mM 2-ME and 1 M NaCl). The tissue slices were gently shaken in buffer B for 10 min. The washing fluid that contained extracellular proteins was taken and used as the first IWF. The resulting tissue slices were further washed four times with 10 ml of buffer B to remove all of the proteins in the intercellular space. AI activity was not detected in the final IWF when tissue had been washed four times. The IWFs that containd AI activity were combined and used for experiments. The tissue slices after this washing procedure were homogenized with buffer B, and the supernatant after centrifugation at 10,000 × *g* for 10 min was dialyzed against buffer B to give the intracellular fraction.

Extraction of acid invertase—AI was isolated from tomato pericarp tissue as described previously (Endo et al. 1990).

Assay of enzymatic activity—AI activity was assayed in a reaction mixture that consisted of 4% sucrose, 0.1 M sodium acetate buffer (pH 4.5) and an aliquot of enzyme solution in a total volume of 1 ml. The reaction mixture was incubated at 30°C for 15 min. Reducing groups formed in the reaction mixture were measured as described previously (Endo et al. 1990). Polygalacturonase activity was assayed as described previously (Yoshida et al. 1984). *α*-Mannosidase activity was assayed in 100 mM sodium succinate (pH 5.0) that contained 1 mM *p*-nitrophenyl *α*-D-mannopyranoside (0.5 ml) at 30°C for 60 min. The reaction was terminated by addition of 0.8 ml of 1 M Na_2CO_3. Free *p*-nitrophenol formed by the enzymatic reaction was quantitated from its absorbance at 405 nm. One unit of activity was defined as the amount of enzyme that hydrolyzed 1 μmol of substrate in 1 min under the conditions used.

Immunoblot analysis—Proteins were separated by SDS-PAGE on a 10% gel and transferred to a nitrocellulose sheet as described by Burnette (1981). The AI on the sheet was detected with antibodies raised against tomato AI in rabbit as described previously (Endo et al. 1990).

Results and Discussion

Localization of AI of tomato fruits—S and CW forms

Table 1 Localization of acid invertase in tomato fruits

Fraction	AI activity		Polygalacturonase activity		α-Mannosidase activity	
	(units (g fr wt)$^{-1}$)	(%)	(units (g fr wt)$^{-1}$)	(%)	(units (g fr wt)$^{-1}$)	(%)
Exp-1						
Soluble fraction	0.08	2.3				
Cell wall-bound fraction	3.44	97.7				
Exp-2						
Intracellular fraction	0.01	0.5	0.038	1.9	20.7	93.5
Intercellular washing fluid	1.9	99.5	1.93	98.1	1.4	6.5

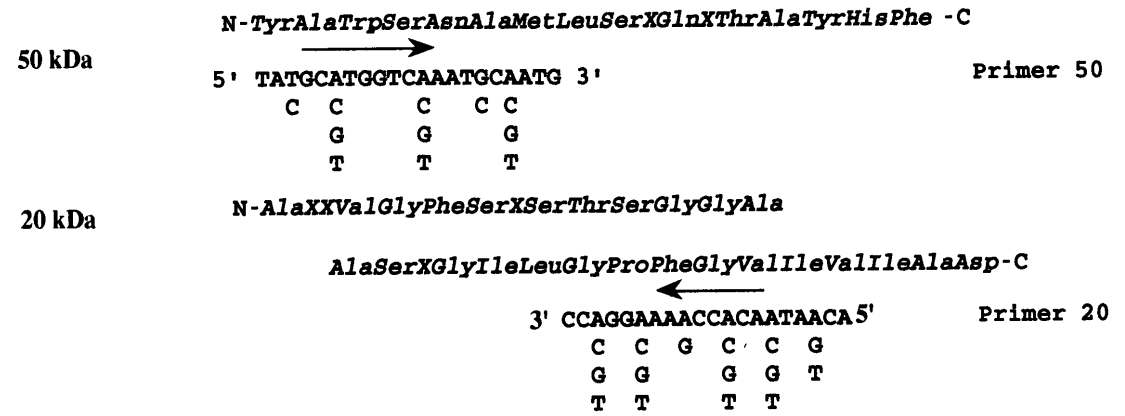

Fig. 1 The N-terminal amino acid sequence of the 50-kDa and 20-kDa polypeptides from a preparation of acid invertase from tomato fruits. Sequences of synthetic oligonucleotide mixtures used as primers for amplification of a cDNA by PCR are shown under the corresponding amino-acid sequences. X shows the position of unidentified amino-acid residue.

of AI were isolated from the pericarp tissue of tomato fruits. As shown in Table 1, the majority of the AI activity was contained in the CW fraction, but 2% of the extractable AI activity was detected in the S fraction. The optimum pH for AI in the S fraction and in the CW fraction was 4.5 and 5, respectively. To determine the localization of tomato AI, proteins that were located in the intercellular space (cell wall) were released by treatment with a buffer that contained 1 M NaCl (IWF). IWF contained most of the activity of polygalacturonase, which is known to be localized in the cell-wall area (Table 1, Exp-2). By contrast, only 6.5% of the α-mannosidase, which is present within cell, was detected in the IWF (Table 1, Exp-2). These results indicate that the IWF obtained by our procedure contained proteins that are present only in the cell-wall region without any serious contamination by intracellular enzymes. Most of the AI activity (99.5%) in the tomato fruits was found in the IWF (Table 1, Exp-2). These results show that the tomato AI was truly localized in the cell-wall fraction and only a trace of AI activity was detected in soluble and intracellular fractions. S and CW AI may be different proteins but the possibility that S and CW AI are products of a common gene cannot be excluded at present.

Cloning of a cDNA for acid invertase of tomato fruits —When the preparation of purified AI that had contained

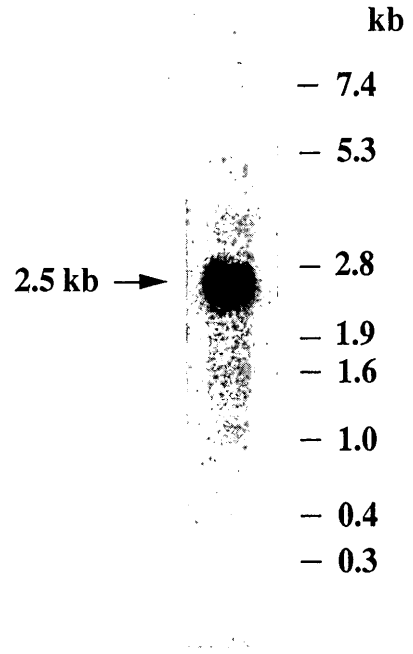

Fig. 2 RNA blot analysis of mRNA for acid invertase from ripe tomato fruits. The poly(A)$^+$-RNA isolated from ripe tomato fruits was fractionated on a formaldehyde-containing gel and transferred to a nylon membrane. The blot was probed with the tomato cDNA.

a single 50-kDa polypeptide (Endo et al. 1990) was analyzed by SDS-PAGE after storage at −80°C for five years, an additional polypeptide of 20 kDa was found. Since the 20-kDa polypeptide appeared to be a product of the degradation of purified AI, the N-terminal amino acid sequences of both the 50-kDa and 20-kDa polypeptides were determined. The N-terminal amino acid sequence of the 20-kDa polypeptide showed a high degree of similarity to the sequence from Ser^{492} to Asp^{520} of invertase from mung bean (Arai et al. 1992). The result indicated that the 20-kDa poly-

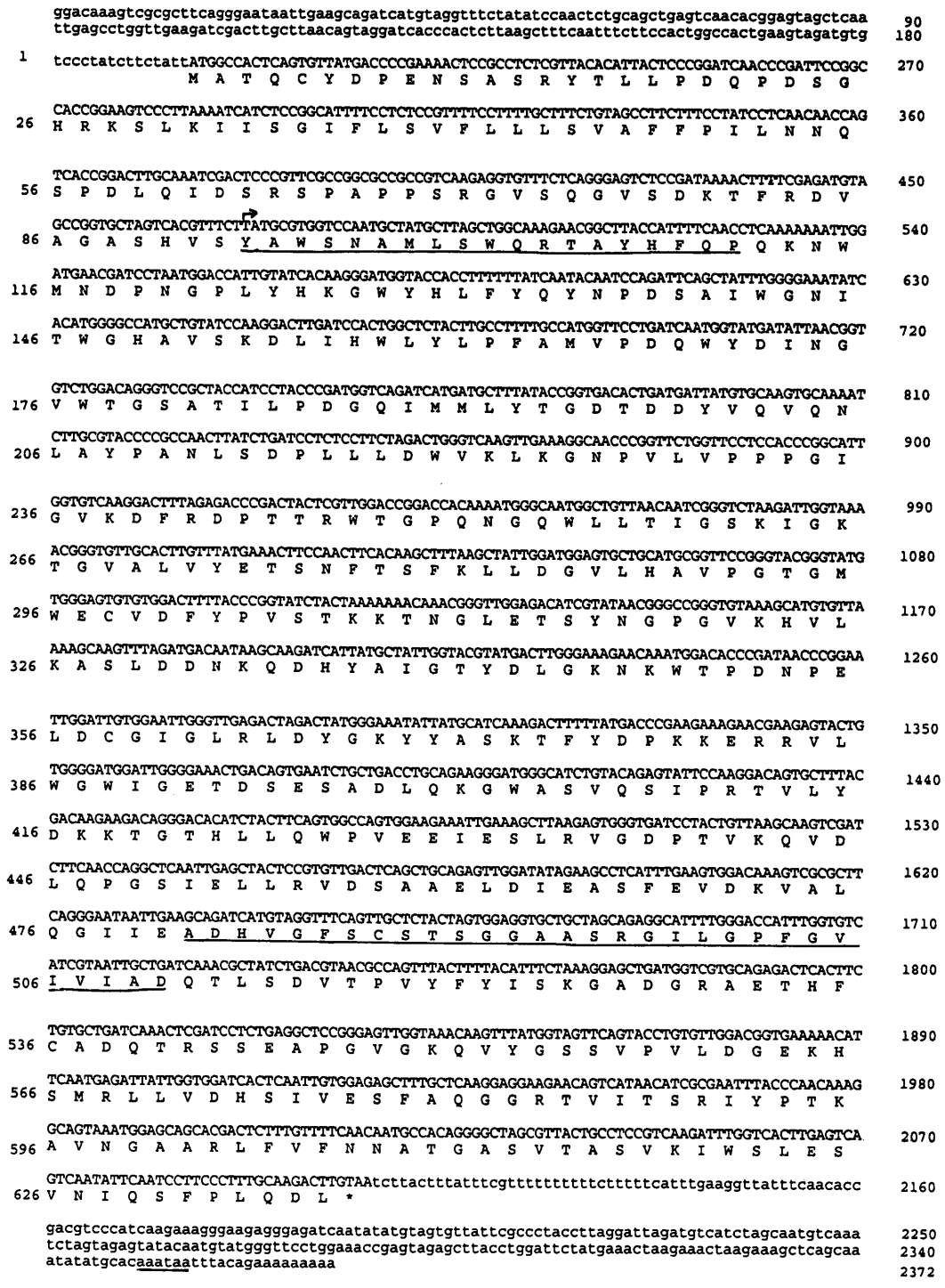

Fig. 3 The nucleotide and deduced amino-acid sequences of a cDNA (TAI1) for acid invertase from tomato fruits. The coding region is given in capital letters. The N-terminus is shown by a hooked arrow. Partial amino-acid sequences determined from the 50-kDa and 20-kDa polypeptides are underlined.

peptide was a fragment of tomato AI located closer to the C-terminal than the partial sequence of the 50-kDa polypeptide. Thus, two oligonucleotides, synthesized as shown in Fig. 1, were used as primers to amplify a fragment of tomato cDNA by PCR. A fragment of cDNA of about 1.2 kbp was amplified and was of the size expected from that of mung bean AI. The 1.2-kbp fragment hybridized to an RNA of about 2.5 kb from ripe tomato fruits (Fig. 2). By screening 10^5 colonies from a cDNA library with the 1.2-kbp fragment as probe, we isolated a cDNA of 2.3 kbp (TAI18-22). However, TAI18-22 was found not to contain the entire coding region of AI. The cDNA library was again screened with a 600-bp fragment obtained from the 5′-side of TAI18-22, and 12 positive clones were isolated from 10^5 colonies. Partial restriction maps of 12 clones were all identical, but two clones (TAI1 and TAI2) contained cDNA that was about 200 bp longer than TAI18-22. The complete nucleotide sequence and the deduced amino-acid sequence of TAI-1 are presented in Figure 3. The cDNA of 2,363 bp contained an open reading frame that encodes a protein of 636 amino acids with a calculated molecular mass of 70,161 daltons. The ATG triplet at positions 196–198 was assigned as the site of initiation of translation because there was an in-frame termination codon (TAG) at positions 124–126. The nucleotide sequence around the first ATG triplet (ATTATGG) is in good agreement with the eukaryotic consensus sequence for a site of initiation of translation (Kozak 1983). In the 3′ noncoding region, a consensus sequence for a polyadenylation signal (AAATAA) was found at a site 13 nucleotides upstream (nucleotides 2351 to 2356) from the poly(A) tail.

The deduced amino-acid sequence of AI from tomato

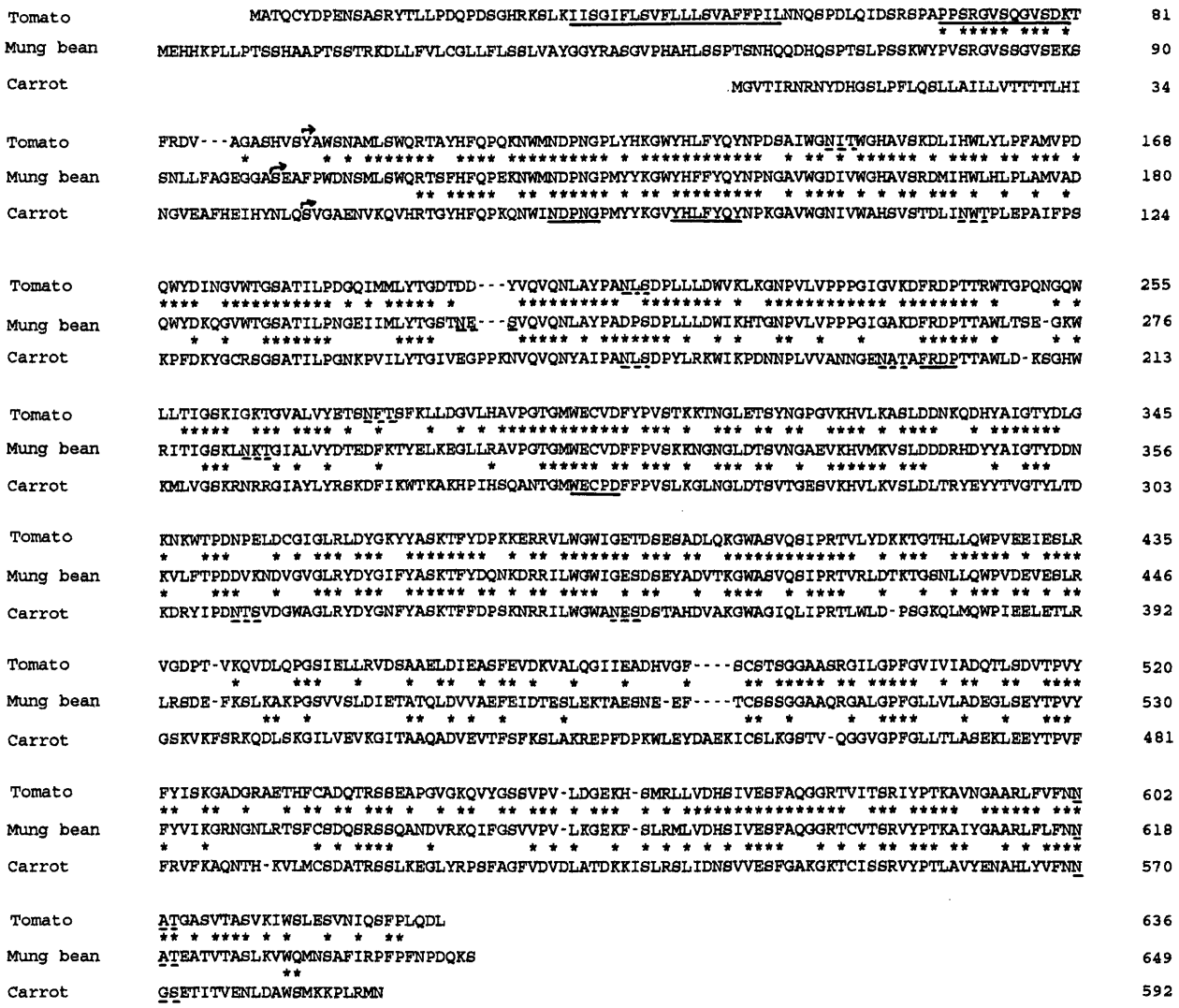

Fig. 4 Comparison of amino acid sequences of acid invertases from tomato, mung bean and carrot. Identical amino-acid residues are marked with asterisks. A potential site of N-glycosylation is shown with dashed underlining. The N-termini are shown by hooked arrows. Underlining indicates the β-fructosidase motif (NDPNG), the catalytic domain (WECVD) and the sequences descussed in the text.

fruits was 38 amino acid longer than that of AI from carrot, but it was 14 amino acid shorter than that of AI from mung bean seedling (Fig. 4). The amino acid residue at the N-terminus of purified AI was Tyr^{93} (hooked arrow in Fig. 4). These results indicate that AI of tomato fruits is produced as a precursor that has a long leader sequence of 92 amino acid residues (Figs. 3, 4). The first 68 amino acids of the leader sequence contain a hydrophobic region (Ile^{32} to Leu^{52}) that is also found in AI from carrot (Sturm and Chrispeels 1990) and mung bean (Arai et al. 1992). The sequence of the hydrophobic region showed no similarities at all to the corresponding region of the enzymes from carrot and mung bean. The next 13 amino acids (Pro^{68} to Lys^{80}) were very similar to those in the mung bean enzyme (Pro^{87} to Lys^{99}). The sizes of the mature forms of the AIs were very similar among the three AIs (544 amino acids for AIs from tomato and carrot and 548 amino acids for AI from mung bean), and the extent of the similarities in amino acid sequence between the mature form of tomato AI and the enzymes from carrot and mung bean was 45% and 65%, respectively. The predicted protein of tomato AI contains four potential sites of glycosylation (Fig. 4, dashed underlining). Both the 50-kDa and 20-kDa polypeptides of AI bound to concanavalin A. Therefore, at least a site of glycosylation in the most C-terminal region must be N-glycosylated with high-mannose type of glycan. The relative location of this glycosylation site is conserved among the three plant AIs. The β-fructosidase motif (Asn^{117}-Asp-Pro-Asn-Gly), which is a conserved peptide domain common to all β-fructosidases (Sturm and Chrispeels 1990), and a catalytic domain specific for β-fructosidases (Trp^{296}-Glu-Cys-Val-Glu,; Martin et al. 1987) are found in AI from tomato fruits, but the proline residue in the AI-specific catalytic domain has been replaced by Val in AI from tomato fruits as has that in the AI from mung bean seedlings. In addition, two short sequences of Tyr^{129}-His-Leu-Phe-Tyr-Gln-Tyr and Phe^{240}-Arg-Asp-Pro, which are conserved in β-fructofuranosidases, were found in the AI from tomato fruits. After the present work had been completed, a report of the nucleotide sequence of a cDNA for AI from tomato fruits appeared (Klann et al. 1992). A direct comparison of the nucleotide sequences of the two cDNAs for tomato AI indicates that there is 98.9% homology. The amino acid sequence deduced from the sequence of our cDNA was identical to the published sequence with the exception of six amino acid residues, where Klann et al. found Phe^{223}, Ala^{246}, Asp^{313}, Glu^{515}, Leu^{516} and Ala^{620}. The difference in amino acid sequence between the two cDNAs might be a result of the difference in the variety of tomato that was used.

Changes in the levels of mRNA for AI in tomato fruits during ripening—Mature green tomato fruits contained low levels of AI activity, but the AI activity increased greatly as fruits ripened (Fig. 5-A). The level of the AI polypeptide was also low at the mature green stage but it increased rapidly as the activity increased (Fig. 5-B). These results show that the increase in AI activity is due to increased amounts of the enzyme protein. The level of AI

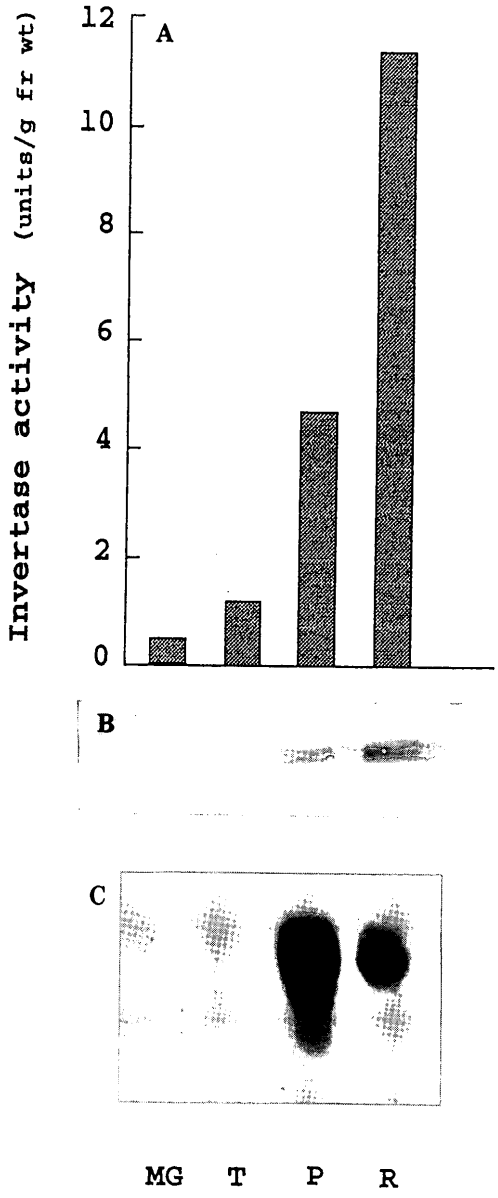

Fig. 5 Changes in the levels of activity, the AI polypeptide and the mRNA for AI in the pericarp tissue of tomato fruit during ripening. AI and total RNA were prepared from pericarp tissues of tomato fruits at designated stages. Immunoblot analysis was performed as described in Materials and Methods. Total RNA was fractionated by agarose gel electrophoresis and then transferred to a nylon membrane. The blot was probed with the tomato cDNA. MG, T, P and R indicate mature green, turning, pink and ripe stages, respectively. A, AI activity; B, levels of AI polypeptide as determined by immunoblot analysis; C, levels of AI mRNA as determined by RNA blot analysis.

mRNA was very low at the mature green stage, but it increased rapidly after the turning stage. It reached a maximum at the pink stage and decreased gradually thereafter (from the pink to the ripe stage; Fig. 5-C). The results demonstrate that the increase in activity of AI, observed in tomato fruits during ripening, is regulated by expression of the gene. The time-course of the pattern of gene expression is similar to that of the gene for polygalacturonase (Grierson et al. 1987), and the gene for AI may be a ripening-related gene. However, the function of the enzyme in fruit ripening remains to be determined.

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