

# Plant Enolase: Gene Structure, Expression, and Evolution

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**Enolase genes were cloned from tomato and *Arabidopsis*. Comparison of their primary structures with other enolases revealed a remarkable degree of conservation, except for the presence of an insertion of 5 amino acids unique to plant enolases. Expression of the enolase genes was studied under various conditions. Under normal growth conditions, steady-state messenger and enzyme activity levels were significantly higher in roots than in green tissue. Large inductions of mRNA, accompanied by a moderate increase in enzyme activity, were obtained by an artificial ripening treatment in tomato fruits. However, there was little effect of anaerobiosis on the abundance of enolase messenger. In heat shock conditions, no induction of enolase mRNA was observed. We also present evidence that, at least in *Arabidopsis*, the hypothesis that there exists a complete set of glycolytic enzymes in the chloroplast is not valid, and we propose instead the occurrence of a substrate shuttle in *Arabidopsis* chloroplasts for termination of the glycolytic cycle.**

## INTRODUCTION

Enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) is a ubiquitous enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, the only dehydration step in the glycolytic pathway. The enzyme has been purified and/or the genes have been cloned from diverse sources: *Escherichia coli* (Weng et al., 1986), *Bacillus subtilis* (D. Vereecke, unpublished data; Verma, 1989), yeast (Brewer, 1981; Chin et al., 1981; Holland et al., 1981), *Drosophila* (Bishop and Corces, 1990), *Xenopus* (Segil et al., 1988), chicken (Russell et al., 1986), Peking duck (Wistow et al., 1988), and several mammals (Sakimura et al., 1985b; Forss-Petter et al., 1986; Lazar et al., 1986; Giallongo et al., 1990). The yeast enzyme is by far the best characterized and its crystal structure has been determined at high resolution (Lebioda et al., 1989; Stec and Lebioda, 1990). Both *cis* elements and *trans*-acting factors governing metabolic regulation and coordination of the two structural genes *ENO1* and *ENO2* have been analyzed (Cohen et al., 1986, 1987; Holland et al., 1987; Machida et al., 1989; Brindle et al., 1990). Moreover, enolase was found to be identical to the heat shock protein HSP48 and, hence, important in thermal tolerance and growth control of yeast (Iida and Yahara, 1985). In higher vertebrates, three different isozymes of enolase have been

described ( $\alpha$ ,  $\beta$ , and  $\gamma$ ), and their cell type and developmental specificity have been demonstrated (Forss-Petter et al., 1986; Giallongo et al., 1986). Interestingly, the lens structural protein  $\tau$ -crystallin has been identified as  $\alpha$ -enolase in different vertebrates (Wistow et al., 1988).

Very little information is available on plant enolases. The enzyme has been purified and partially characterized from potato (Boser, 1959) and spinach (Sinha and Brewer, 1984), but our knowledge on enolase gene and protein structure and its biological relevance in plants remains extremely poor. It is generally accepted that fermentative or anaerobic metabolism is not prevalent throughout the normal life cycle of photosynthetically active cells, but in certain nongreen and anaerobically treated tissues, which are dependent on glycolysis and oxidative phosphorylation for their energy supply (Goodwin and Mercer, 1983), this enzyme becomes important. Moreover, photosynthetic cells have been shown to break down carbohydrates in the dark, mainly by the glycolytic pathway (Goodwin and Mercer, 1983).

In this paper, we present insights on the role of enolase in plant life. Enolase genes and cDNAs were cloned from tomato and *Arabidopsis*. The primary and secondary structures of plant enolases were compared with their bacterial, yeast, and animal counterparts. We also present evidence that the occurrence of two forms of each glycolytic enzyme, a cytosolic and a plastid isoform (Gottlieb, 1982),

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cannot be generalized to include enolase. In addition, we describe the expression of enolases (mRNA and protein) in normal and in stressed plant tissues. Based on these data, we present some general conclusions concerning glycolysis in anaerobic and heat shock-treated plant tissue.

## RESULTS

### Isolation and Characterization of Tomato and *Arabidopsis* Enolase Genes

The amino acid sequence of the N terminus and of several tryptic peptides from a 45-kD protein originating from a highly purified tomato 1-aminocyclopropane-1-carboxylate (ACC) synthase preparation revealed about 50% similarity with yeast enolase (Van Der Straeten et al., 1990). Mixed oligonucleotides were synthesized based on the main sequences of peptides P26 and P46 (Figure 1 in Van Der Straeten et al., 1990). A tomato (cv Orlando) cDNA library was constructed in  $\lambda$ gt11, and from 25,000 recombinant clones, we obtained seven hybridizing phages, five of which had an insert size above 1.2 kb. The complete sequence of the largest clone (TEN01) is shown in Figure 1A. The cDNA of 1554 bp contains an open reading frame encoding a polypeptide of 47.7 kD (444 amino acids) with a calculated pI of 5.6, a 5'-untranslated leader of 60 bp, and a trailing sequence of 162 bp. In contrast to what was observed for the yeast enolase genes (Holland et al., 1981), codon usage is unbiased in the tomato gene, with the exception of Lys, where AAG is used in 30/39 cases.

One member of the tomato enolase gene family was isolated from a VNTF Cherry  $\lambda$  Charon 35 genomic library screened with the TEN01 cDNA as a probe. The 3-kb HindIII fragment, which was sequenced (Figure 1A) and designated gTEN02, covers 75% of the coding region (326/444 residues). The intron/exon structure is presented in Figure 1B. The 12 introns invariably contain the GT and AG dinucleotide consensus at their respective 5' and 3' ends (Csank et al., 1990), with the exception of the 5' end of the third intron, where GC occurs. This could inhibit splicing of the intron, implying that gTEN02 might be a pseudogene. The coding regions of gTEN02 and the TEN01 cDNA are 89% identical and thus TEN01 most probably is derived from a different gene because this divergence is not readily accounted for by cultivar differences. However, the amino acid similarity is 96%, as only 14 base substitutions lead to translational changes.

Figure 2 presents a genomic DNA gel blot that confirms the existence of an enolase multigene family in tomato. Hybridization of the TEN01 BglII-EcoRI fragment to total genomic tomato DNA revealed three BglII and three EcoRI restriction fragments, some of which had an intensity higher than one copy equivalent (Figure 2, lanes 4). It would seem, therefore, that the tomato genome contains

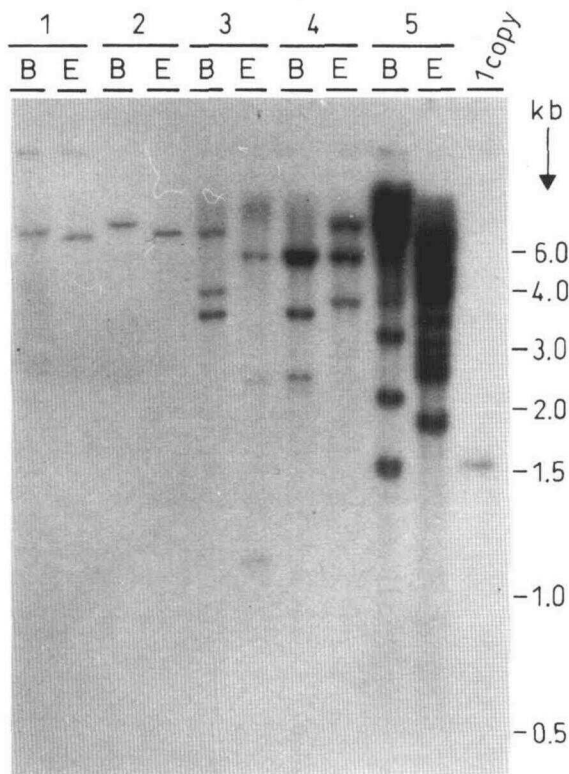
at least three, and probably more, genes encoding enolase. Under high-stringency conditions (65°C, 250 mM phosphate buffer), strong hybridization was observed to *Arabidopsis* (Figure 2, lanes 1 and 2), rice (Figure 2, lanes 3), and tobacco DNA (Figure 2, lanes 5), indicating a high degree of interspecies similarity. Three hybridizing bands were present in the rice lanes, whereas multiple bands were observed with tobacco DNA. Thus, the existence of multiple enolase isoforms appears to be fairly common in the plant kingdom.

In *Arabidopsis*, however, hybridization occurred to a single BglII and EcoRI restriction fragment, both of which were approximately 7 kb in length and had the intensity of one copy equivalent. The unique *Arabidopsis* enolase gene was cloned by hybridization with mixed oligonucleotide probes to about 30,000 colony-forming units from a genomic cosmid library with the mixed oligonucleotide probes (see above). Figure 3A presents the complete sequence of the *Arabidopsis* enolase gene (4553 bp). The sequence covers 1060 bp of the 5'-untranslated region and 786 bp at the 3' end. Putative TATA and CAAT boxes (Joshi, 1987), as well as potential polyadenylation signals (Dean et al., 1986), are underlined. The gene contains 11 introns, all displaying the consensus dinucleotides at their boundaries (Csank et al., 1990) (Figure 3B). The positions of exon boundaries are conserved between tomato and *Arabidopsis*, with the exception of exons 6, 7, and 10 of the *Arabidopsis* gene, which are joining two or three corresponding exons of the tomato gene. Although the plant enolases that we have cloned are most similar to the mammalian  $\alpha$  isoform, there is no conservation of intron organization between the human  $\alpha$  gene (Giallongo et al., 1990) and the *Arabidopsis* or tomato enolase genes. Similarities between the entire tomato and *Arabidopsis* coding regions are 80% at the DNA and 90% at the amino acid level.

Because several glycolytic enzymes are anaerobically inducible and carry the anaerobic responsive element (ARE) core consensus sequence in their 5' end or first intron (for a review, see Dennis et al., 1987), we have looked for its presence in the *Arabidopsis* enolase gene. The essential or core anaerobic region TGGTTT was found in the first intron, 228 bp downstream from the translational start. The surrounding sequence is 62% similar to the 13-bp sequence of the maize alcohol dehydrogenase 1 (ADH1) ARE core consensus, which is also present in the first intron of the maize sucrose synthase gene (Springer et al., 1986).

Because enolase was reported to be heat shock inducible, as are several other glycolytic enzymes in yeast (Iida and Yahara, 1985; Piper et al., 1988), we searched for sequence similarity to the plant consensus heat shock element (HSE) 5'-T-TTC--GAA-A-3' (Schöffl et al., 1989) in the *Arabidopsis* enolase promoter. Maximal similarity was in the region 833-844, where the sequence 5'-TATTCTGCAAAA-3' occurs. Although 75% similar to





**Figure 2.** Genomic DNA Gel Blot Analysis of *A. thaliana* (ecotype C24), *Oryza sativa* (cv Taipei), *L. esculentum* (cv Orlando), and *Nicotiana tabacum*.

Lanes 1, *A. thaliana*, 1  $\mu$ g of total DNA; lanes 2, *A. thaliana*, 1  $\mu$ g of nuclear DNA; lanes 3, rice, 3  $\mu$ g of nuclear DNA; lanes 4, tomato, 5  $\mu$ g of total DNA; lanes 5, tobacco, 20  $\mu$ g of nuclear DNA. B, BglII; E, EcoRI. One-copy reconstruction on the right. Filters were hybridized with a  $^{32}$ P-labeled 1.5-kb BglII-EcoRI fragment of the TENO1 cDNA clone described in Methods. Exposure was for 6.5 hr on flash-sensitized film.

soybean HSE2, this sequence does not correspond entirely to the required hyphenated dyad, and, moreover, it is not interlocked with a second heat shocklike element, another essential requirement for heat shock inducibility (Schöffl et al., 1989).

### Primary and Secondary Structure Comparisons of Enolases

Figure 4 shows an amino acid sequence alignment and secondary structure predictions for prokaryotic (*B. subtilis*; D. Vereecke, unpublished data; Verma, 1989) and different eukaryotic enolases. The eukaryotic sequences are from

*Arabidopsis*, tomato, chicken ( $\beta$  form; Russell et al., 1986), *X. laevis* (Segil et al., 1988), rat ( $\gamma$  form; Sakimura et al., 1985a), human ( $\alpha$  form; Giallongo et al., 1986), and yeast (*ENO1* gene product; Holland et al., 1981).

A number of deductions can be made from inspection of the amino acid alignment. The derived consensus sequence covers all amino acids found in the active site region of yeast enolase (Lebioda et al., 1989), with the exception of Lys<sup>345</sup> and Lys<sup>396</sup>, which are replaced by Gly and Glu, respectively, in the chicken  $\beta$  form. The high degree of similarity between enolases is also shown in Table 1, where pairwise comparisons are presented. Plant enolases have a lower similarity when compared with yeast (either *ENO1* or *ENO2*) than do higher animals compared with yeast. When compared with animal sequences, plant enolases have the highest similarity to the  $\alpha$  form. The sequence around Tyr<sup>46</sup> in the  $\alpha$  form, which was shown to be phosphorylated in Rous sarcoma virus-infected chicken embryo fibroblasts (Cooper et al., 1984), is identical to the same region in plants. Whether this is of any significance in plant cells remains to be elucidated. Finally, higher eukaryotic enolases have an increased number of cysteines (>5), all of which appear at positions different from the single cysteine in *Bacillus* or yeast enolase.

Another striking feature is the occurrence of a 5-amino-acid insertion unique to plant enolases (QNEWG, residues 101 to 105). The influence of this insertion on the secondary structure was investigated. Predicted structures are presented in Figure 4, "averaging" four different methods. The pentapeptide unique to plants is part of a predicted coil region, which is also predicted in coil for the other enolases and corresponds to a coil region in a model based on high-resolution x-ray crystallography of yeast enolase (Lebioda et al., 1989). Therefore, the pentapeptide is expected not to influence the tertiary structure significantly. However, it is possible that the 4 amino acids, adjacent to the insertion, extend the neighboring  $\alpha$  helix.

The validity of the predictions was supported by the fact that the secondary structures of different enolases are in good agreement over aligned positions. It was further tested by "averaging" the secondary structures of all species ("join pred." in Figure 4) and comparing them with the model of Lebioda et al. (1989). The alignment in Figure 4 shows a reasonable agreement between both models, although the position and/or the extent of a secondary structure is often missed by a few residues. Helices are more faithfully predicted than sheets (e.g., the last  $\beta$  sheet is not seen in our averaged secondary structure). The predictions are in any case more reliable than those generated using the Chou-Fasman method (Chou and Fasman, 1978; data not shown) and seem to be more accurate than previous predictions of enolase secondary structure (Sawyer et al., 1986).







**Table 1.** Percent Amino Acid Similarity between Different Prokaryotic and Eukaryotic Enolases

	1	2	3	4	5	6	7	8	9
1		43.0	42.6	43.2	41.6	42.6	39.5	43.0	43.0
2			95.4	51.1	53.5	60.8	59.6	59.4	61.3
3				50.4	53.0	59.7	57.5	58.0	60.1
4					89.9	64.5	58.7	63.6	65.4
5						66.8	59.8	64.0	66.6
6							78.3	82.0	87.8
7								80.8	81.5
8									83.2
9									

1, *B. subtilis*; 2, yeast *ENO1* gene product; 3, yeast *ENO2* gene product; 4, *A. thaliana*; 5, tomato; 6, *X. laevis*; 7, chicken  $\beta$  form; 8, rat  $\gamma$  form; 9, human  $\alpha$  form. Percent identity was calculated by the PC/Gene program, Release 6.01 (A. Bairoch, University of Geneva, Switzerland).

### Absence of Plastid Enolase in *Arabidopsis*

There is a single nuclear-encoded enolase gene in *Arabidopsis* because nuclear DNA gel blots (Figure 2, lanes 2) showed one band and total genomic DNA also yielded one hybridizing band (the weak signal above is from non-digested DNA remaining in the wells; Figure 2, lanes 1). Any chloroplast-encoded enolase must have a highly diverged sequence. Moreover, sequence comparison between TENO1 and both the tobacco and liverwort chloroplast genome sequences (IntelliGenetics Suite, Release 5.37, IntelliGenetics, Mountain View, CA) did not reveal any significant similarity. Indeed, in those cases that have been studied, cytosolic and plastid glycolytic enzymes are all encoded by the nuclear genome (Weeden and Gottlieb, 1980; Cerff and Klopstech, 1982; Gottlieb, 1982; Shih et al., 1986).

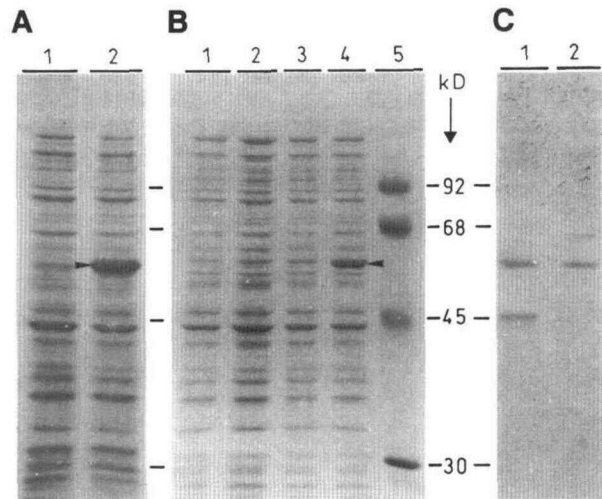
In addition, no homology was found between the enolase N-terminal amino acid sequence and the consensus chloroplast target sequence (Schmidt and Mishkind, 1986; von Heijne et al., 1989). This, together with the fact that the *Arabidopsis* enolase N terminus is very similar to the *Bacillus* and yeast N-terminal sequences (Figure 4), supports the idea that the *Arabidopsis* enolase polypeptide is not imported into the chloroplast. Further evidence in support of this conclusion is based on our inability to detect enolase in purified chloroplasts by either activity or immunoassays (data not shown). Both assays were positive on total leaf extracts. The arguments presented above strongly indicate that *Arabidopsis* chloroplasts lack at least part of the glycolytic pathway.

### Expression and Activity of Tomato Enolase in *E. coli*

Tomato enolase was expressed in *E. coli* in two different ways. A translational fusion was made to the  $\phi 10$  gene in

the pT7-7 expression vector. The hybrid polypeptide lacks 11 N-terminal amino acids from enolase, which are replaced by 9 amino acids encoded by the polylinker of the pT7-7 vector. Figure 5A shows that the recombinant protein, induced by temperature shift of a K38(pGP1-2/pT7-7-TENO1) culture, migrates at 56 kD in denaturing polyacrylamide gels (SDS-PAGE; Laemmli, 1970). The denatured recombinant product was recovered from preparative Laemmli gels and used to generate a polyclonal antiserum in rabbits.

Furthermore, the complete coding region of TENO1 was expressed under control of the Tac promoter in *E. coli*. The isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)-induced polypeptide migrated as a 56-kD protein on SDS-PAGE (Figure 5B). This molecular mass discrepancy (the calculated value is 48 kD) is difficult to explain because it cannot be due to glycosylation. We found, however, that the enolase protein from a partially purified tomato extract also migrates at an anomalously high molecular mass. The enolase antibody recognizes both a 56-kD and a 45-kD product on a protein gel blot of this tomato extract (Figure 5C). The 45-kD polypeptide also corresponds to enolase because its N-terminal sequence is 45% homologous to



**Figure 5.** Expression of Recombinant Tomato Enolase in *E. coli*.

(A) Expression of the translational fusion T7-7-TENO1 in K38(pGP1-2) cells. Lane 1, negative control (insert in reverse orientation); lane 2, temperature-induced recombinant enolase. The induced 56-kD protein is indicated by the arrowhead.

(B) Expression of intact recombinant enolase in JM109 cells. Lane 1, cells transformed with pBTac2 without IPTG; lane 2, after IPTG induction; lane 3, cells transformed with pBTac2-TENO1 without IPTG; lane 4, after IPTG induction; lane 5, molecular mass markers. The induced 56-kD protein is indicated by the arrowhead.

(C) Immunoblot analysis of tomato enolase. Lane 1, partially purified tomato enolase extract; lane 2, extract from induced K38(pGP1-2/pT7-7-TENO1).

yeast enolase and identical to amino acids 50 to 59 in the tomato polypeptide predicted from the TENO1 clone. The 45-kD product is probably the result of artificial processing during purification.

Interestingly, the intact recombinant protein was active in *E. coli* extracts. Enolase activities were on average fivefold higher than in the noninduced controls (specific activities 4690 units/ $\mu\text{g}$  of protein and 950 units/ $\mu\text{g}$  of protein in controls). In contrast, K38(pGP1-2) cells transformed with the construct with the modified N terminus did not show activities above basal endogenous levels. This was confirmed when the same plasmid was transformed to BL21(DE3) cells and induced at low growth temperature with IPTG to avoid thermal denaturation. We conclude that an intact N-terminal region is essential for plant enolase activity.

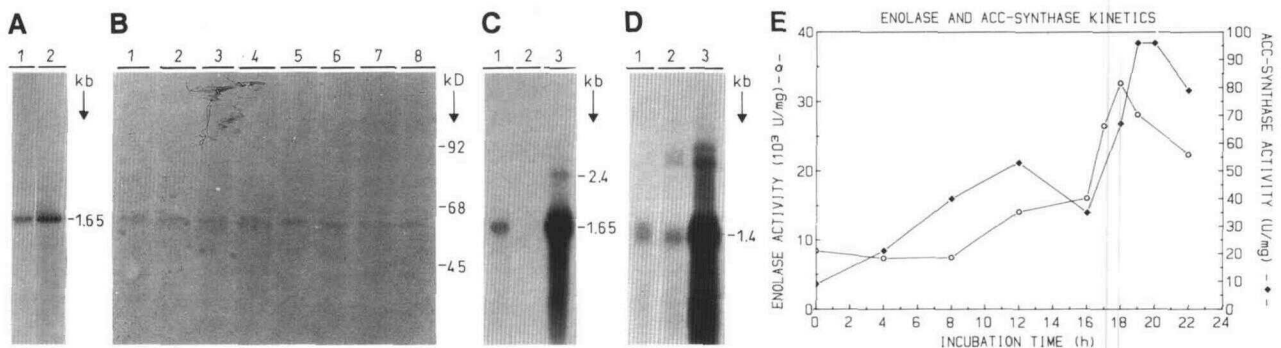
### Expression of Enolases in Plants: Tissue and Developmental Specificity

Expression of enolase genes was examined in different plant tissues. First we investigated differences in mRNA levels in photosynthetic and nongreen tissue. Figure 6A presents an RNA gel blot of tomato leaf and root tissue probed with the TENO1 cDNA. Because the leaf sample contains 10  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA and the root lane 100  $\mu\text{g}$  of total RNA, which is approximately equivalent to 1  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA, and because the signal in root is about

twofold stronger than in leaf, we estimate that the expression in roots is 10-fold to 20-fold higher than in leaves (comparable signals were obtained from two independent hybridization experiments with different samples). Similar results were obtained in *Arabidopsis* (data not shown).

In addition, we investigated whether this difference in steady-state mRNA levels is also observed for enolase protein. Figure 6B presents a protein gel blot of protein from different tissues of *Arabidopsis*. The antibody recognized a 56-kD protein in *Arabidopsis*. The level of enolase seemed reasonably constant in all tissues. Because glycolytic activity is expected to be higher in nonphotosynthetic tissue (Goodwin and Mercer, 1983), we compared enolase activities in *Arabidopsis* roots and leaves. Specific activities in roots were as high as 107,000 units/mg, whereas leaf activities were at least fivefold lower. It is concluded that tissue-specific regulation of enolases is complex and, although occurring at the transcriptional or post-transcriptional level, apparently also involves post-translational controls.

A second aspect of interest is the developmental control of enolase expression. It has been proposed that the carbon flux through glycolysis increases during fruit ripening (Stitt et al., 1986). The expression pattern of enolase mRNA at different stages of tomato fruit ripening was analyzed (Figure 6C). Surprisingly, the 1.6-kb messenger was present in green fruit but undetectable in pink fruit. However, when pink fruits were artificially ripened by a combined treatment with LiCl, hormones, and wounding (Van Der Straeten et al., 1990), the enolase mRNA was



**Figure 6.** Tissue-Specific and Development-Specific Expression of Enolase in Tomato and *Arabidopsis*.

(A) RNA gel blot analysis of tomato leaf and root. Lane 1, tomato leaf tissue, 10  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA; lane 2, tomato root tissue, 100  $\mu\text{g}$  of total RNA. The TENO1 BglIII-EcoRI fragment was radiolabeled and used as a probe. Exposure was overnight on flash-sensitized film.

(B) Immunoblot analysis of enolase in different tissues of *Arabidopsis*. Each lane contains approximately 100  $\mu\text{g}$  of total protein. Lane 1, flowers; lane 2, stem; lane 3, leaf; lane 4, unripe pods; lane 5, ripe pods; lane 6, roots; lane 7, etiolated seedlings; lane 8, green seedlings.

(C) RNA gel blot analysis of tomato fruit at different stages. Each lane contains 10  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA. Lane 1, green tissue; lane 2, pink tissue; lane 3, artificially ripened pink tissue. A random primed probe of the TENO1 BglIII-EcoRI fragment has been used. Exposure was for 12 hr on flash-sensitized film.

(D) As in (C), but probed with a tomato ADH partial cDNA. Exposure was for 24 hr on flash-sensitized film.

(E) Time course of induction of enolase and ACC synthase by artificial ripening.



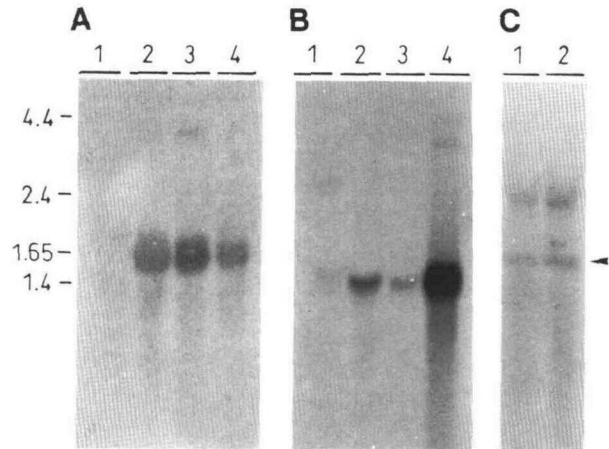
increased at least 30-fold over green tissue. This significant rise in mRNA was caused by the chemicals and the wounding treatment because a water control did not lead to any increase of enolase messenger compared with pink tissue (data not shown). When the same blot was probed with a partial tomato ADH cDNA (D. Van Der Straeten, unpublished results), we observed a similar expression pattern with the exception of pink tissue, where a signal was detected of equal intensity to green fruit (Figure 6D), indicating the presence of certain glycolytic mRNA species in this ripening stage.

The induction of enolase mRNA during the artificial ripening process was concomitant with an increase in enzyme activity, as shown in Figure 6E. The kinetics of enolase induction were very similar to ACC synthase kinetics (Van Der Straeten and Van Montagu, 1990); nevertheless, the activity increase for enolase was much lower (threefold versus 50-fold). However, the highest enolase activities were found in green tissue (52,000 units/mg), despite the fact that it did not have the most elevated mRNA levels.

### Expression of Plant Enolases in Stress Conditions

Anaerobic stress is known to induce the first and last enzymes of the glycolytic pathway at the mRNA level and, in general, also at the protein level (Dennis et al., 1987; Bailey-Serres et al., 1988). For certain intervening glycolytic enzymes like enolase, small increases in activity were observed in maize (Bailey-Serres et al., 1988). We have investigated whether this is preceded by a rise in enolase mRNA accumulation. Figure 7A presents an RNA gel blot that shows the effect of anaerobiosis in seedlings and roots of tomato. As a control for the hypoxic induction, we analyzed the same samples with a partial tomato ADH cDNA as a probe (D. Van Der Straeten, unpublished results) (Figure 7B). A fair increase in enolase mRNA occurred in submerged tomato seedlings. There was, however, no detectable increase of messenger in waterlogged tomato roots. By contrast, there was a strong induction of ADH messenger, both in hypoxically treated seedlings (at least 10-fold) and in roots (at least 30-fold), confirming the validity of the test conditions. Figure 7C presents an RNA gel blot of mRNA from submerged *Arabidopsis* seedlings probed with the TENO1 cDNA. A low increase in mRNA level was observed.

In yeast, HSP48 is identical to enolase (Iida and Yahara, 1985), although no effect on the *ENO1* promoter was demonstrated (Uemura et al., 1986). Therefore, we tested whether enolase is heat shock-inducible in plants. The effect of heat shock on enolase mRNA accumulation was tested in tomato leaves and roots. Figure 8 shows a significant decrease in enolase message in both tissues after 1-hr exposure to the elevated temperature; the level



**Figure 7.** Expression of Enolase and ADH under Anaerobic Stress.

**(A)** RNA gel blot of submerged etiolated tomato seedlings and waterlogged tomato roots. Each lane contains 100  $\mu$ g of total RNA. Lane 1, etiolated seedlings; lane 2, after 20 hr submergence; lane 3, root tissue; lane 4, root tissue after 40 hr waterlogging. A  $^{32}$ P-labeled BglII-EcoRI fragment of TENO1 was used as a probe. Exposure was for 72 hr on flash-sensitized film.

**(B)** As in **(A)**, but probed with a tomato ADH partial cDNA. Exposure was for 24 hr on flash-sensitized film.

**(C)** RNA gel blot of submerged etiolated *Arabidopsis* seedlings. Both lanes contain 50  $\mu$ g of total RNA. Lane 1, control; lane 2, after a 20-hr submergence period. Exposure was overnight on flash-sensitized film. The arrowhead indicates the enolase mRNA signal.

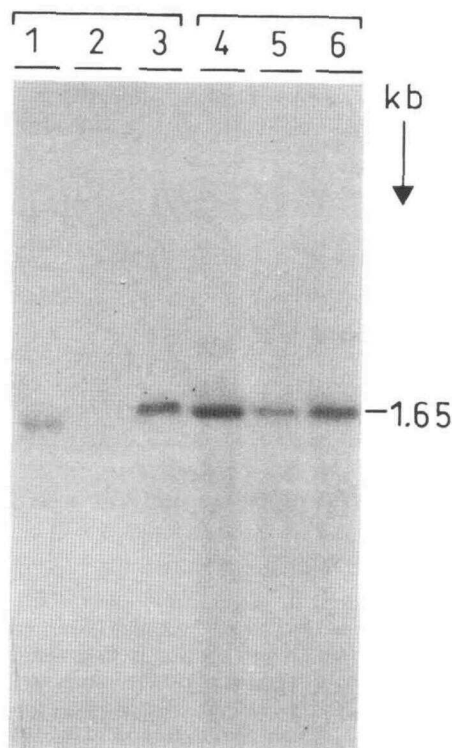
mRNA sizes are indicated in kilobases on the left.

was at least partially restored after 2 hr. This result was confirmed in a separate experiment where samples were taken after 1 hr and 3 hr (data not shown). Consistently, no increase in enolase activity was found (data not shown). This transient response is observed for most cellular proteins when a plant is subjected to heat shock (Kimpel and Key, 1985; Lindquist, 1986).

## DISCUSSION

### A Most Conserved Polypeptide

In this report we described the cloning of enolases from *Arabidopsis* and tomato. To our knowledge, enolase is the first glycolytic protein from which the primary structure is elucidated in all kingdoms and for which a detailed tertiary structure model exists.



**Figure 8.** RNA Gel Blot Analysis of Tomato Enolase under Heat Shock Conditions.

The TENO1 BgIII-EcoRI fragment was radiolabeled and used as a probe. Lane 1, 10  $\mu$ g of leaf poly(A)<sup>+</sup> RNA; lane 2, after 1 hr at 40°C; lane 3, after 2 hr at 40°C; lane 4, 100  $\mu$ g of total RNA from root; lane 5, after 1 hr at 40°C; lane 6, after 2 hr at 40°C. Exposure time was 17 hr on flash-sensitized film.

From a primary structure comparison of enolases throughout evolution, the following can be concluded. Eukaryotic enolases are, on average, 42% identical to the prokaryotic *Bacillus* enzyme. This high degree of conservation is a property of most glycolytic enzymes, which is also reflected by their exceptionally low rate of evolution, about as low as cytochrome *c* (Fothergill-Gilmore, 1986). Obviously, most of the residues located in or near the active site (Lebioda et al., 1989) remain unchanged. Strikingly, however, yeast enolases have a significantly lower similarity to their plant counterparts (52%) than to their animal counterparts (60%). This might imply that the yeast and plant progenitor genes have diverged earlier than yeast and animal ancestral genes. This idea is consistent with the multikingdom tree proposed by Sogin (1989).

Furthermore, the primary structure comparison revealed the existence of an insertion of 5 amino acids unique to the plant enolases. A secondary structure prediction, averaging four different methods, allowed us to conclude

that this pentapeptide is residing in a coil region and, therefore, probably has very little influence on tertiary folding. This was according to our expectations because it was demonstrated recently that yeast enolase, as three other glycolytic enzymes and nine other enzymes, possesses an  $\alpha/\beta$  barrel structure, which nonetheless is a variation on the common theme (Lebioda and Stec, 1988). All of these enzymes have a core of mostly parallel  $\beta$  sheets surrounded by  $\alpha$  helices. This is a thermodynamically extremely stable structure, perhaps arising from convergent evolution (Fothergill-Gilmore, 1986). Therefore, it was anticipated that the plant enzyme would not be different from other enolases from a secondary and tertiary structure point of view.

Finally, we evaluated the influence on tertiary structure of the presence of 5 cysteine residues in plant enolase, as opposed to a single cysteine in *Bacillus* and yeast. It seems highly unlikely that the cysteines in plant enolase would form intramolecular disulfide bridges, thereby possibly distorting the structure proposed for yeast enolase (Lebioda et al., 1989). When their positions are superimposed on a model as presented by these authors, it appears that the cysteines reside on opposing sides of the barrel, too distant from each other to allow bridge formation. Interestingly, the cysteine in position 408 in plants, which is also conserved in all other eukaryotic enolases, is apparently spatially near the position of the unique cysteine in yeast (position 247), residing close to the active site (Lebioda et al., 1989). It has been proven that chemical modification of this residue in yeast leads to loss of activity (Oh et al., 1973). However, further investigations will have to demonstrate that its role in plants and other eukaryotes might be played by a cysteine located elsewhere in the chain.

### Plant Enolase Usually Appears as Multiple Isoforms

In most organisms, the glycolytic pathway has evolved by gene duplication, giving rise to different isozymes (Fothergill-Gilmore, 1986). In this way, selection may have produced enzymes optimal for the chemical differences and metabolic demands of specific tissues. In most cases described for plants, these isozymes include at least one cytosolic and one plastid isoform (Gottlieb, 1982; Shih et al., 1986). The existence of enolase isozymes has been demonstrated on the protein level in developing castor oil seeds (Miernyk and Dennis, 1982). Our data support this fact on the gene level for several plant species. A genomic DNA gel blot from four different species revealed the existence of at least three gene copies for three of them. Unless these are pseudogenes or sequences split by long introns, the results indicate the presence of three genes in rice, three or four in tomato, and an even more numerous gene family in tobacco. The only exception to the presence

of multiple genes was *Arabidopsis*, where a single band was detected on genomic DNA gel blots in both nuclear and total DNA preparations. This demonstrates that there is no requirement for multiple enolase genes to meet the demands of eukaryotic existence.

### ***Arabidopsis* Plastids Lack Part of the Glycolysis**

As mentioned above, glycolytic enzymes in general possess at least two isoforms, a cytosolic form and a plastid form. In those cases that have been studied, both types are encoded by the nuclear genome (Weeden and Gottlieb, 1980; Cerff and Kloppstech, 1982; Gottlieb, 1982; Shih et al., 1986). Shih et al. (1986) provided convincing evidence for the symbiotic theory, which predicts that genes for the chloroplast enzymes have been transferred from the genome of a prokaryotic symbiont to the nucleus. Several reports, however, have mentioned the absence of certain glycolytic activities in chloroplasts (Stitt and apRees, 1979) or nongreen plastids (Macdonald and apRees, 1983; Journet and Douce, 1985; Frehner et al., 1990). Our data indicate on the gene level that certain plant species, as demonstrated in *Arabidopsis*, indeed have an incomplete set of glycolytic enzymes in their plastids. This conclusion was based on several lines of evidence.

First, *Arabidopsis* possesses a single, nuclear-encoded gene for enolase. Furthermore, this gene encodes a polypeptide whose N-terminal region is highly homologous to the *Bacillus* and yeast enolase N terminus and, moreover, has no homology to the consensus chloroplast target sequence (von Heijne et al., 1989). Finally, both the enolase protein and its activity are absent in *Arabidopsis* chloroplasts. Therefore, it seems not only that the *Arabidopsis* chloroplast enolase gene was lost at some point in evolution, but also that *Arabidopsis* plastids must bypass this step in glycolysis. The consequence of this fact is different for chloroplasts and nongreen plastids. In chloroplasts, glycolytic enzymes are not involved mainly in glycolysis, but have their function in photosynthetic carbon fixation (dark phase). Enolase is one of the few steps that is not involved in the Calvin-Benson cycle and, therefore, there is no essential need for it in the chloroplast, although enolase activity has been demonstrated in isolated pea chloroplasts, where it plays a role in carbohydrate breakdown in the dark (Stitt and apRees, 1979). The situation is different in nongreen plastids, which have to convert hexose phosphates to pyruvate for fatty acid synthesis and, therefore, need to be able to transform 2-phosphoglycerate in phosphoenolpyruvate. Although we did not check enolase activity in nongreen plastids, we suggest the existence of a substrate-product shuttle to bypass enolase in *Arabidopsis* nongreen plastids. Such a shuttle system was proposed in some other cases, although, to the best of our knowledge, never for enolase (Stitt and

apRees, 1980; Journet and Douce, 1985; Frehner et al., 1990).

### **Complex Controlling Mechanisms Regulate Enolase Expression**

In nonphotosynthetically active cells, glycolysis plays a role in energy metabolism. In plants, however, the situation is different because plant cells rely primarily on photosynthesis for their energy supplies. The question remains: what are the tissues and the circumstances under which glycolysis proceeds in plant cells? Messenger RNA and enzyme activity analyses in tomato and *Arabidopsis* demonstrated that enolase expression is more prevalent in roots than in green tissue. This is consistent with the higher demand for energy from glycolysis in nongreen tissue. However, a protein gel blot of different tissues of *Arabidopsis* showed that higher activities in root tissue are not a simple reflection of increased amounts of protein. The fact that there is very little variation on the protein level indicates possible involvement of post-translational modification. Further investigations will be needed to solve this issue.

Several studies have reported an increase of glycolytic intermediates during the ripening process or upon exposure to ethylene or cyanide (Chalmers and Rowan, 1971; Solomos and Laties, 1974; Stitt et al., 1986). We have analyzed how this influences mRNA accumulation and enzymatic activities of enolase in tomato fruit.

In green fruits, the prevalence of messenger RNA was lower than in roots. Corresponding enzyme activities were reduced by a factor of two. Surprisingly, enolase mRNA was undetectable in pink fruit, and enzyme activity was approximately fivefold lower than in green fruit. It is conceivable that the enolase protein is formed at the mature green stage and subjected to metabolic controls in later stages of ripening. Activation of pre-existing enzymatic activity has been suggested for glycolysis in general (Solomos and Laties, 1974). Interestingly, when pink fruits were ripened artificially, enolase mRNA accumulated to high levels, at least 20-fold higher than in green fruit. Enzyme activities increased by a factor of three, consistent with a threefold increase in glycolytic carbon flux during ripening, as reported earlier (Solomos and Laties, 1974). There are three possible explanations for the fact that the high prevalence of message was not accompanied by an equal increase in enzymatic activity. First, it may reflect the post-translational controls postulated earlier. Second, it is possible that efficiency of translation is much lower in the artificially ripened tissue than in mature green fruit. This type of regulation has been reported for sucrose synthase in maize (McElfresh and Chourey, 1988; Rowland et al., 1989; Taliercio and Chourey, 1989). The alternative explanation is based on the fact that lithium ions have

been reported to inhibit enolase (Kornblatt and Musil, 1990), even at concentrations 10-fold lower than applied in the artificial ripening process. Due to the inhibition by lithium, the actual active enzyme concentration is dramatically lowered and could trigger a feedback mechanism controlling transcription. There are, however, no data available on the irreversibility of this inhibition.

In conclusion, it is clear that both tissue and developmental specificity of enolase in plants exist and that these are regulated by a complex mechanism including controls at the level of transcription, and possibly also at the levels of translation and post-translation.

### Enolase, as Other Intervening Glycolytic Enzymes, Shows Low Responsiveness to Anaerobiosis

Under conditions of low oxygen tension, plants have been shown to shift their carbohydrate metabolism from an oxidative to a fermentative pathway. On the cellular level, a specific set of polypeptides is synthesized, the so-called anaerobic polypeptides (Sachs et al., 1980), whereas the synthesis of aerobic proteins is discontinued. Several of the anaerobic polypeptides are associated with glycolysis (for a review, see Dennis et al., 1987). Their induction occurs both at the transcriptional level and at the level of enzymatic activity. However, there is a significant difference in the level of induction for the first and the last enzymes of the glycolytic pathway on the one hand and for the intervening glycolytic enzymes on the other hand. The latter enzymes are generally induced to a fairly low level (twofold) or not at all (Kelley and Freeling, 1984; Bailey-Serres et al., 1988). The strongly hypoxically induced genes have been shown to contain a consensus sequence (ARE), with a core hexanucleotide TGGTTT essential for anaerobic induction (Dennis et al., 1987; Walker et al., 1987; Olive et al., 1990), present in the promoter region and also in the first intron, as for sucrose synthase (Springer et al., 1986). The ARE core was found in the first intron of the *Arabidopsis* enolase gene. A second region in the maize *Adh1* ARE containing the hexanucleotide CGGTTT (Walker et al., 1987) was present in the enolase promoter, however, only 27 nucleotides upstream from the putative TATA box, not accompanied by the essential core element and, therefore, probably not functional. It was tested whether the presence of the ARE core in intron 1 is sufficient for anaerobic induction of enolase in *Arabidopsis*. A very low induction was observed in submerged *Arabidopsis* seedlings (approximately twofold). It was concluded that the ARE core element necessarily needs to be present in the promoter region to be functional. Earlier studies on maize glyceraldehyde-3-phosphate dehydrogenase (GAPDH), where the core element was found at the required position from the transcriptional start but anaerobic induction could not be demonstrated (Martinez

et al., 1989), implied that the ARE core element is a necessary but not a sufficient condition for high anaerobic induction.

Low anaerobic transcriptional induction of enolase was observed in tomato and compared with tomato ADH induction in the same conditions. In tomato seedlings, a fair increase in steady-state messenger was observed. By contrast, enolase root mRNA levels remained constant. In both tissues, however, ADH mRNA was induced at least 10-fold to 20-fold under the same hypoxic treatment. An expression pattern similar to enolase was reported for GAPDH in rice seedlings and roots under anaerobiosis (Rivoal et al., 1989). The results reported here support the hypothesis that intermediate steps in the glycolytic pathway may be less responsive to anaerobiosis than are the initial and final parts of the pathway.

### Enolase Is Not a Heat Shock Protein in Plants

Iida and Yahara (1985) proved that yeast enolase is identical to HSP48 and is involved in thermal tolerance and growth control in yeast. Heat shock induction was also reported for other yeast glycolytic proteins, as phosphoglycerate kinase and GAPDH (Piper et al., 1988). We tested whether enolase might be heat shock inducible in plant cells. When tomato roots and leaves were exposed to elevated temperatures, enolase mRNA accumulation patterns were similar to those found for most cellular proteins. An initial decrease in mRNA after the first hour of heat exposure was followed by a restoration of the normal level of expression after 2 hr to 3 hr of heat shock. This kinetic, indicating that growth resumed after a temporary pause (Lindquist, 1986), was found in leaves as well as in roots. As expected, we did not find a significant homology to consensus plant HSE (Schöffl et al., 1989) interlocked with a second dyad element, required for heat shock inducibility. It was concluded that plant enolase is not a heat shock-inducible protein.

## METHODS

### Plant Material, Growth Conditions, and Stress Treatments

*Arabidopsis thaliana* (ecotype C24) plants were grown at 22°C and 80% humidity. Tomato plants (*Lycopersicon esculentum* cv Supersonic) were grown at 24°C and 80% humidity. Tomato fruits (green or pink; cv Orlando) were obtained from a local market.

Artificial ripening conditions were as described elsewhere (Van Der Straeten et al., 1990).

Hypoxic induction of 13-day-old dark-grown *Arabidopsis* and tomato seedlings was by complete submergence for 20 hr in 10 mM Tris-HCl, pH 7.0, containing 75 µg/mL chloramphenicol, as

described by Springer et al. (1986). When applied to 1.5-month-old tomato plants, waterlogging conditions were simulated by placing the plants in a 100-liter tank filled with water to 2 to 3 cm above the soil surface for 40 hr at 21°C.

Heat shock was done by incubation of 2-month-old tomato plants at 40°C for 1, 2, or 3 hr.

### Assay for Enolase Activity

Activity measurements for enolase were performed in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM MgCl<sub>2</sub> and 2.5 mM 2-phospho-D-glycerate, as modified from Westhead (1966). Incubations were at 25°C for 10 min. The absorbance at 230 nm was measured against a blank without substrate. A calibration curve was established for phosphoenolpyruvate, the linearity of the reaction was confirmed for each sample, and each value is the average of three independent measurements. One unit is defined as the amount of enzyme that converts 1 nmol of substrate per hour at 25°C.

### Isolation of Chloroplasts and Protein Extraction

Chloroplasts were isolated from *Arabidopsis* leaves essentially according to Mullet and Chua (1983). Mitochondrial contamination was checked by a cytochrome *c* oxidase assay (Darley-Usmar et al., 1987).

Extraction of protein from different plant tissues was as described by Bauw et al. (1987). Protein measurements were according to Bradford (1976).

### Peptide Sequencing

Proteins separated on 10% Laemmli gels (Laemmli, 1970) were electroblotted onto coated glass fiber sheets, and the N-terminal amino acid sequence of the protein of interest was determined as described previously (Van Der Straeten et al., 1989). Total tryptic digestion, separation, and sequencing of tryptic peptides were as reported earlier (Van Der Straeten et al., 1989, 1990).

### Secondary Structure Predictions

Secondary structure predictions were based on four different methods: the GOR method (Garnier et al., 1978), the homolog method (Levin et al., 1986), the GGBSM method (Gascuel and Golmard, 1988), and the method of Rooman and Wodak (1988). The methods were applied for each enolase separately; a residue was considered likely to adopt a helical, an extended strand, or a coil conformation if at least three methods were in agreement. A joint prediction was performed by aligning all enolases and assigning to each position the conformation that was occurring most frequently.

### RNA Isolation, Construction, and Screening of a Tomato cDNA Library

The construction of a tomato pericarp cDNA library, prepared from RNA induced by artificial ripening, was according to Van Der Straeten et al. (1990). Approximately 25,000 plaque-forming units were plated and screened on Hybond N (Amersham Corp.) using mixed oligonucleotide probes (100 pmol). The sequences of the oligonucleotides are: 5'-(A/G)TC ICC (A/G)TT IAT IAC (A/G)TT (A/G)AA IGC IGG IAC IGG-3' (33-mer, derived from peptide P46) and 5'-TT IGG IGC (A/G)AA ICC ICC (C/T)TC (A/G)TC ICC IAC-3' (29-mer, derived from P26). Hybridization was performed at 40°C in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 0.5% SDS, 0.1 mg/mL denatured herring sperm DNA. Filters were washed twice in 2 × SSC for 30 min at 40°C, followed by 2 × SSC, 0.1% SDS for 30 min at 40°C.

### Genomic Libraries

A cosmid library of *A. thaliana* ecotype Columbia was kindly provided by Dr. Neil Olszewski (University of Minnesota). About 30,000 colony-forming units were screened using the same conditions as for the tomato cDNA library. A λ Charon 35 genomic library of the tomato cultivar VNTF Cherry was a gift from Dr. Robert Fischer (University of California, Berkeley). Two hundred thousand plaque-forming units were screened with a tomato enolase cDNA (designated TENO1) BglII-EcoRI random primed probe. Hybridization and washes were at 65°C.

### Subcloning and DNA Sequence Analysis

The tomato cDNA inserts were subcloned as EcoRI fragments in pUC19. A full-length subclone of 1554 bp was named TENO1. A 7.5-kb fragment containing the entire coding sequence of the unique *Arabidopsis* enolase gene (gAENO) was subcloned in the EcoRI site of pBR325, and a 1.8-kb HindIII subfragment was inserted into pUC18. Subclones of the above-mentioned inserts were sequenced according to Maxam and Gilbert (1977). A 3.0-kb HindIII fragment of a tomato genomic enolase clone (gTENO2) was subcloned in pUC18 and sequenced with internal primers (Sanger et al., 1977).

### Nucleic Acid Hybridization Analysis

Nuclear DNA was prepared according to Jofuku and Goldberg (1988). Total DNA preparation was mainly as described by Dellaporta et al. (1983), followed by a CsCl gradient. The amounts of DNA loaded on gel were correlated with the genome size of the different species used. DNA gels were blotted onto Hybond N (Amersham) and probes labeled by random priming according to the manufacturer's specifications (Amersham). Hybridization was carried out at 65°C as described (Van Der Straeten et al., 1990).

RNA for gel blotting was prepared and purified as reported (Rodrigues-Pousada et al., 1990), and the poly(A)<sup>+</sup> fraction was obtained on oligo(dT)-cellulose (Sambrook et al., 1989). Six-percent formaldehyde gels were blotted on Hybond N (Amersham).

RNA gel blot hybridization was at 42°C in a buffer described previously (Van Der Straeten et al., 1990).

### Expression in *Escherichia coli*, Antibody Production, and Immunoblotting

Plant enolase cDNA was expressed in *E. coli* to obtain high amounts of recombinant protein for subsequent antibody production. Two different strategies were used. A 1.5-kb blunted BglII-EcoRI fragment of TENO1 was subcloned in a blunted BamHI site of the expression vector pT7-7 (Tabor and Richardson, 1985) and then transformed into K38(pGP1-2) cells containing the T7 RNA polymerase gene controlled by a temperature-inducible promoter. The recombinant protein, formed after induction at 42°C, lacks 11 amino acids at the N terminus that are replaced by 9 amino acids from the pT7-7 plasmid. The same construct was also transformed into BL21(DE3) cells (Studier and Moffatt, 1986) to allow induction by IPTG at 22°C.

The second method employed a plasmid constructed by ligation of a synthetic linker coding for the first 11 amino acids of tomato enolase and a TENO1 BglII-PstI fragment into pBTac2 (Boehringer Mannheim). JM109 cells were grown at 28°C to an  $A_{600}$  value of 0.5 in LB medium containing triacillin (50 µg/mL); IPTG was added to 1 mM, and the cells were further incubated for 2 hr. Bacteria were harvested and sonicated in a 50 mM potassium phosphate buffer, pH 7.3, supplemented with 1 mM MgCl<sub>2</sub>. Enolase activities were measured versus noninduced controls.

Two New Zealand White rabbits were immunized with the truncated plant enolase produced in K38(pGP1-2/pT7-7-TENO1) and separated on an SDS-polyacrylamide gel. About 30 µg of protein was injected, first with complete Freund's adjuvant and then three additional times with incomplete adjuvant. Immunoblotting was according to Towbin et al. (1979) with a 1:1000-fold diluted primary antiserum and an antirabbit alkaline phosphatase conjugate as a secondary antibody (Sigma). Under these conditions, the antiserum was able to reveal 10 ng of the recombinant protein but did not cross-react with purified yeast or rabbit enolases (Sigma).

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