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Characterization of a cDNA encoding 5-aminolevulinic acid dehydratase in tomato (*Lycopersicon esculentum* Mill.)

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Summary. A full-length cDNA clone encoding tomato (*Lycopersicon esculentum* Mill.) 5-aminolevulinic acid dehydratase (ALAD) was isolated and characterized. The primary structure predicts a 430-amino acid precursor which comprises a 41.7 kDa, 388-amino acid mature protein and a 47-amino acid transit sequence. The tomato primary sequence shows extensive homology to those of pea and spinach. Southern analysis indicated that 1 to 2 copies of the ALAD gene are present in the tomato genome. Northern blot analysis shows differential expression in various tomato organs, and constitutive developmental expression in tomato fruits.

Abbreviations: ALA, 5-aminolevulinic acid; ALAD, 5-aminolevulinic acid dehydratase (EC 4.2.1.24)

Introduction

Chlorophyll and heme biosynthesis occur via a common pathway from 5-aminolevulinic acid (ALA) to protoporphyrin IX (Beale and Weinstein 1990). 5-aminolevulinic acid dehydratase (ALAD; EC 4.2.1.24), the second enzyme in this pathway, is synthesized in the cytoplasm and is transported into the plastids, where it catalyzes the condensation of two molecules of ALA into porphobilinogen, a pyrrole compound (Richards 1993, Smith 1988). ALAD has been isolated and characterized in a number of animal, plant, and bacterial species (reviewed in Leeper 1991), and recently, cDNAs encoding ALAD have been isolated and described in pea (Boese et al. 1991) and spinach (Schaumburg et al. 1992). ALAD activity increases during chloroplast development that occurs following illumination of etiolated radish cotyledons (Shibata and Ochiai 1976) and this increase in activity was shown to be phytochrome-mediated (Kasemir and Masoner 1975, Tchuinmogne et al. 1989), as was the increase in ALAD protein (Tchuinmogne et al. 1989). In light, ALAD may act as a control point in limiting chlorophyll synthesis via its competitive inhibition by 4,5-dioxovalerate, an intermediate in the synthesis of 5-aminolevulinic acid (Kotzabasis et al. 1989).

Chlorophyll synthesis and turnover are important aspects of tomato fruit development and ripening. Current once-over mechanical harvest of tomatoes results in about a 10 to 30% loss of production because green (immature) tomatoes are not usable. As a preliminary step to understanding the regulation of ALAD and the role ALAD may play in the process of chlorophyll synthesis and turnover in fruits in general, we report the isolation and characterization of a cDNA encoding ALAD in tomato.

Materials and Methods

Plant materials. Tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) plants were grown in a greenhouse (20°C night/24°C day) with supplementary lighting. Flowers were harvested at anthesis, fruits at 10, 45, and 55 days post-anthesis, and leaf, stem, and root samples six weeks after sowing. All samples were stored at -80°C until used for RNA extraction. Additional leaf samples were lyophilized, ground into powder, and stored at -20°C until used for genomic DNA extraction.

cDNA library screening. A λZAP® II cDNA library of young tomato (cv. VFNT cherry) fruit (obtained from Dr. Wilhelm Gruissem, U.C., Berkeley) was screened with a ^{32}P-labelled 1.35-kb *Eco*RI *Pisum sativum* ALAD cDNA insert (Boese et al. 1991) as a probe. Filters were prehybridized and hybridized in 50% (v/v) formamide, 50 mM Na-phosphate buffer, pH 7.0, 1.0 mM EDTA, 2.5X Denhardt's solution (1X Denhardt's: 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 800 mM NaCl, 0.125 mg/ml salmon sperm DNA, and 0.05% (w/v) SDS at 42°C. Filters were washed once for 45 min in 2X SSPE (1X SSPE: 0.15 NaCl, 0.25 M NaH_2PO_4, 25 mM Na_2EDTA), 0.1% SDS at 23°C, twice for 15 min in 1X SSC (0.15 M NaCl, 0.015 M Na-citrate), 0.1% SDS at 23°C, and once for 15 min in 0.1X SSC, 0.1% SDS at 45°C. Filters were exposed to x-ray film with an intensifying screen at -80°C for 96-120 h. Following plaque purification, six positive clones were isolated and their pBluescript® SK- phagemids were excised according to manufacturer's protocols (Stratagene Cloning Systems, La Jolla, CA). The longest insert was subcloned into pGEM®-11Zf(+) (Promega Corp., Madison, WI). Automated dideoxy sequencing (Sanger et al. 1977) on both strands, using SP6, T7, and synthetic oligonucleotide primers, was performed at the Iowa State Nucleic Acid Facility. DNA sequences were analyzed using the GCG program (General Computer Corp., Inc., Madison, WI). The nucleotide sequence for the tomato ALAD cDNA clone has been submitted to the GenBank Nucleotide Sequence Database under the accession number L31367.

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Southern analysis. Genomic DNA was extracted from 'Rutgers' tomato leaves according to the method of Rogers and Bendich (1985). DNA (10μg) was digested with appropriate enzymes, separated by electrophoresis on a 1.0% agarose gel, denatured, and transferred to a nylon membrane in 25 mM Na-phosphate buffer. Membranes were prehybridized and hybridized in 50% formamide, 6.7X SSC, 25 mM Na-phosphate, 0.04% SDS, and 0.125 mg/ml salmon sperm DNA. The ^{32}P-labelled 1.8-kb *Eco*RI/*Xho*I insert of the tomato ALAD clone was used as a probe. Membranes were washed twice for 15 min in 1.0X SSC, 0.1% SDS at 65°C, once for 15 min in 0.1X SSC, 0.1% SDS at 65°C, then exposed to film for autoradiography.

Northern analysis. Total RNA was extracted from various tissues (Dix and Rawson 1983), separated on a 1.4% agarose gel containing 5 mM methyl mercury hydroxide (Thomas 1980), and blotted onto a nylon membrane. Before blotting, gels were stained with ethidium bromide and ribosomal RNAs were visualized under UV light to confirm that equal amounts of RNA were loaded in all lanes. Membranes were prehybridized and hybridized in 50% formamide, 1 M NaCl, 10% (w/v) dextran sulfate, 1.0% SDS, and 0.1mg/ml salmon sperm DNA. The plasmid containing the insert was linearized with *Eco*RI and used as a template to generate an antisense RNA probe using the Riboprobe® system (Promega Corp., Madison, WI) with SP6 RNA polymerase. Membranes were washed twice for 5 min in 2X SSC, 0.1% SDS at 23°C, twice for 15 min in 0.1X SSC, 0.1% SDS at 65°C, and then exposed to film for autoradiography.

Results and Discussion

The nucleotide sequence of the tomato ALAD cDNA consists of 1775 bp, including 51 bp of 5′-untranslated sequence, 1290 bp of coding region, and 434 bp of 3′-untranslated sequence. A single large open reading frame encodes 430 amino acids (Fig. 1). The putative cleavage site I-R-A⇓-S (Fig. 1) of the tomato transit sequence was deduced by comparison with the spinach amino acid sequence (Schaumburg et al. 1992). This motif fits the general requirements for peptidase cleavage sites (Perlman and Halvorson 1983) and is similar to motifs identified in chloroplast targeting sequences of other species, including pea ALAD (Boese et al 1991), white pine and loblolly pine NADPH:protochlorophyllide oxidoreductase (Spano et al. 1992), and pea porphobilinogen deaminase (Spano and Timko 1991, M. Timko, unpublished results *in* Spano et al. 1992). Cleavage at this position would predict a transit peptide of 47 amino acids, and a mature protein of 383-amino acids having a molecular mass of 41,727 Da. This calculated molecular mass is in accordance with those of *Rhodopseudomonas spheroides* (van Heyningen and Shemin 1971) and *Erythrobacter* sp. strain OCh 114 (Shioi and Doi 1988) and with that determined from a spinach ALAD cDNA (40.1 kDa) (Schaumburg et al. 1992), but is less than that determined by SDS-PAGE for spinach (Liedgens et al. 1980). The tomato transit peptide has a number of features common to other chloroplast transit sequences, including a net positive charge, presence of proline in the central area, abundance of alanine, and absence of tryptophan and tyrosine (Phua et al. 1989, Schmidt and Mishkind 1986). Homology occurs among the tomato, pea, and spinach transit sequences only in the area containing the processing site (Fig. 1, position 56-59).

In contrast, with the exception of the immediate N-terminal end, the mature peptide of tomato shows a high degree of homology to those of pea (90.1%) and spinach

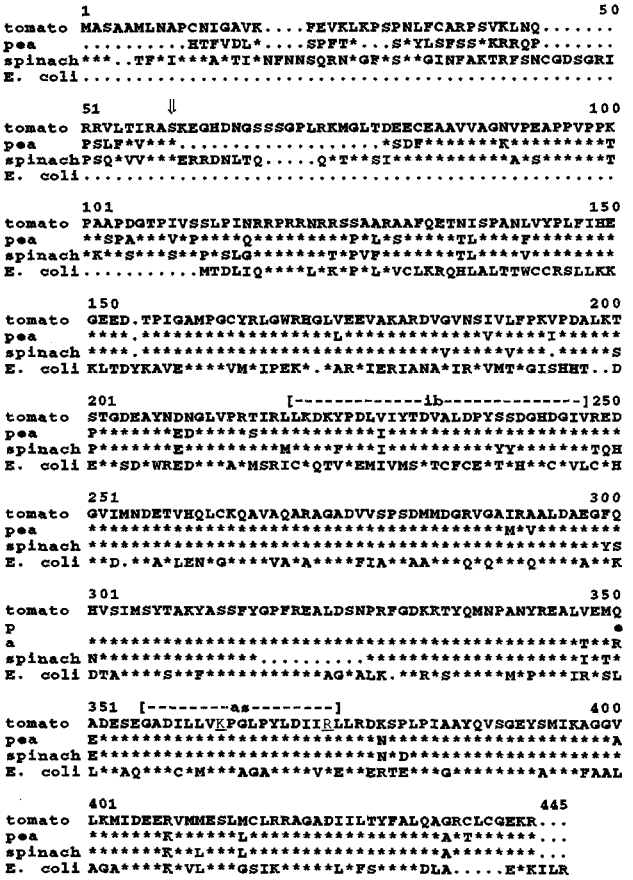

Fig. 1. Comparison of the deduced amino sequence of tomato ALAD with those of pea, spinach, and *E. coli*. Identical amino acids are indicted by asterisks (*). Gaps, indicated by periods (.), were used to optimize the alignment. The arrow indicates the putative cleavage point of the transit sequence. The ion binding and active sites are indicated by [---ib---] and [---as---], respectively. The lysine and arginine residues within the active site region (described in the text) are underlined at positions 363 and 373, respectively.

(85.3%). All three plant species show high homology to *E. coli* (Echelard et al. 1988) in the active site region (Fig. 1, position 355-374) described by Boese et al. (1991) and the adjacent portions of the peptide, but are much less homologous to *E. coli* in other portions of the peptide, including the putative ion-binding region (Fig. 1, position 220-248) (Schaumburg et al. 1992). As in spinach (Schaumburg et al. 1992) and pea (Boese et al. 1991), the tomato ion-binding region lacks the cysteine residues that are present in *E. coli* and that are believed to be involved in binding the Zn^{2+} ion (Wetmur et al. 1986). As noted by Schaumburg et al. (1992) for spinach, additional aspartic acid residues are present in this region in both the tomato and pea peptides, and these may be involved in binding Mg^{2+} ion. The active site regions of all four peptides possess the lysine residue (Fig. 1, position 363) involved in Schiff base formation (Gibbs and Jordan 1986) and an arginine residue (Fig. 1, position 373) essential for substrate conversion (Liedgens et al. 1983).

Southern blot analysis was performed by hybridizing the

1.8 kb tomato ALAD insert to total genomic DNA from tomato leaves (Fig. 2). The hybridization pattern indicates that 1-2 copies of ALAD are present in the tomato genome.

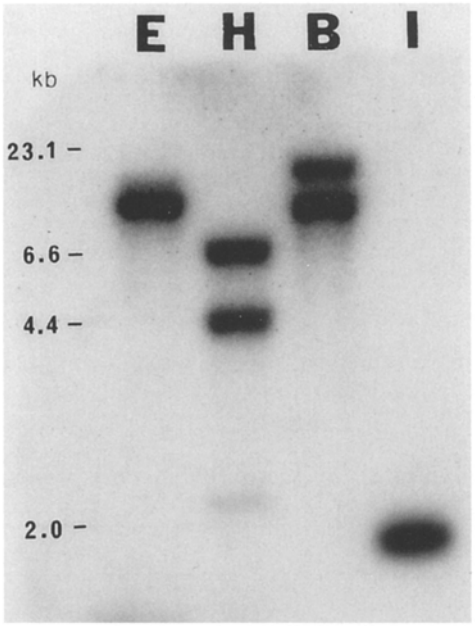

Fig. 2. Southern hybridization of tomato genomic DNA with the 1.8 kb tomato ALAD cDNA fragment. DNA (10 µg) was digested with *Eco*RI (lane E), *Hin*dIII (lane H), or *Bam*HI (lane B). Lane I contains 10 pg of the ALAD cDNA insert, which, relative to the mass of genomic DNA per lane, is equivalent to 1 to 2 copies of the insert.

To study both the tissue-specific expression of ALAD in tomato plant organs and the temporal expression in tomato fruits, northern blot analysis was performed on total RNA isolated from leaves, stems, roots, flowers, and 10-day, 45-day (breaker stage), and 55-day (red ripe) fruits. Northern analysis (Fig. 3) shows that the ALAD probe hybridized to up to four different sized messages in different tissues. Signals of the expected, 1.8 kb message were highest in stems, roots, and fruits. These results are in direct contrast to those of Boese et al. (1991), who found highest levels of expression in leaves, low levels in stem, and barely detectable levels in roots. These differences may be due to collection of samples at different stages of development or to species differences. Little change in transcript levels were apparent in the fruit samples, showing that ALAD is expressed constitutively at the RNA level, and indicating that ALAD expression may be controlled during translation or protein processing. This is in agreement with findings that showed that levels of ALAD protein and activity change with tissue type and stage of development (Huault et al. 1987, Kasemir and Masoner 1975). An alternative explanation is that a sustained high level of ALAD mRNA is needed for increased heme synthesis during the respiratory climacteric and/or for continued synthesis of chlorophyll, which is quickly turned over. The higher molecular weight transcripts were most abundant in total RNA extracted from leaf tissue. High levels of accumulation were also seen in stem and fruit tissue, but only low levels of accumulation were evident in roots and flowers.

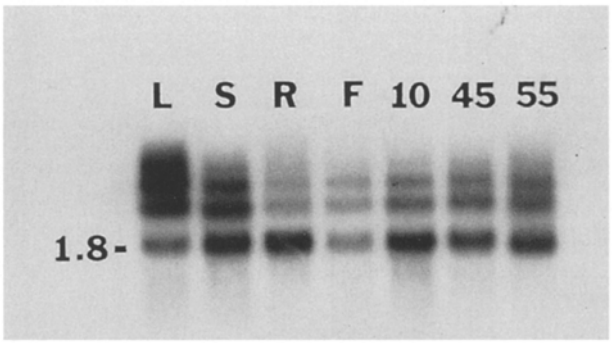

Fig. 3. Northern blot analysis of the steady-state levels of ALAD RNA of tomato leaf (L), stem (S), root (R), and flower (F) tissue, and 10-, 45-, and 55-day tomato fruits. Fifteen micrograms of total RNA were added per lane. The filter was hybridized with a ^{32}P-labelled RNA probe synthesized from ALAD template DNA, in a 50% (v/v) formamide solution at 65 °C. Final wash was in 0.1X SSC, 0.1% SDS at 65 °C.

The observed hybridization to the higher molecular weight transcripts is difficult to interpret. Differences in hybridization intensity were seen in the second band, which corresponds in size to the 25S ribosomal band. The photograph of the RNA gel, however, showed that equal levels of both 18S and 25S rRNAs were present in all samples. Hybridization signals to the higher molecular weight bands were not affected by DNase treatment of samples, and were removed in a similar pattern as those of the 1.8 kb band as the washing temperature was increased progressively to 90 °C (data not presented). In addition, hybridization to higher molecular weight bands was observed when using a ^{32}P-labelled ALAD cDNA insert (data not presented), indicating that the differences were not due solely to greater nonspecific binding of the RNA probe. Nevertheless, the possibility of nonspecific binding to higher molecular weight ribosomal RNAs cannot be dismissed. The higher molecular weight bands may represent different stages of processing of the immature ALAD transcript. The greater abundance of higher molecular weight bands and of total (1.8 kb plus higher-weight species) messages in leaf, stem, and fruit tissue correlates with the greater requirement for chlorophyll and heme synthesis in these tissues. Additional research clearly is needed to characterize the developmental pattern of ALAD expression in different tissues and under different environmental conditions.

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