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Tomato alcohol dehydrogenase
Expression during fruit ripening and under hypoxic conditions

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We have isolated and sequenced a partial tomato alcohol dehydrogenase (*Adh*) cDNA clone. Expression of tomato *Adh* was studied at the messenger RNA level in seedlings, roots, and fruit. High induction was observed under hypoxic conditions, both in tomato seedlings and in roots. In addition, the *Adh* mRNA was present at the mature green and pink stage of the tomato fruit, and was highly induced in late ripening. Moreover, an artificial ripening treatment resulted in at least 50-fold induction compared to the mature green mRNA level. Genomic DNA gel blotting suggested the presence of a multigene family for *Adh* in tomato.

Alcohol dehydrogenase; Anaerobiosis; Glycolysis; Ripening; *Lycopersicon esculentum*

1. INTRODUCTION

Alcohol dehydrogenase (ADH; alcohol:NAD^+ oxidoreductase; EC 1.1.1.1) is a terminal step in glycolysis, leading to ethanolic fermentation under anaerobic conditions. The enzyme from horse liver has been structurally characterized at the three-dimensional level [1]. Plant ADH has been studied in quite some detail at the molecular level. Genes encoding alcohol dehydrogenase have been cloned and sequenced both from monocotyledonous (maize [2]; barley [3]; rice [4,5]) and dicotyledonous species (*Arabidopsis thaliana* [6]; pea [7]; potato [8]). Under hypoxic conditions, plants shift their metabolism from aerobic to fermentative pathways. Alcohol dehydrogenase has been identified as one of the major anaerobic polypeptides (ANPs; [9]), and the *cis*-acting controlling elements responsible for high anaerobic *Adh* mRNA induction have been extensively characterized [10,11].

Recently it has become clear that *Adh* is not only responsive to anaerobic stress, but to a wide range of stresses, including elicitors, salicylic acid, UV light [8], and low temperature treatment [12]. An interesting and so far almost unexplored research area concerns the molecular aspects of glycolysis in general and ADH in particular, during the ripening process of fruits and vegetables. This could be important in storage under controlled and modified atmospheres, where hypoxia could lead to dramatic increases of mRNA and/or protein levels, thus having an impact on the flavour quality of the fruit. In this report, we present cloning of a partial tomato *Adh* cDNA and characterize its expression under anaerobiosis in tomato seedlings and roots as well as in ripening tomato fruit.

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2. MATERIALS AND METHODS

2.1. *Plant material, growth conditions, and anaerobic inductions*

Tomato plants (*Lycopersicon esculentum* cv. Supersonic) were grown at 24°C and 80% humidity. Hypoxic induction of 13-day-old, dark-grown tomato seedlings was by complete submergence for 20 h in 10 mM Tris-HCl, pH 7.0, containing 75 μg/ml chloramphenicol, as described by Springer et al. [13]. When applied to 1.5-month-old tomato plants, waterlogging conditions were simulated by placing them in a 100-liter tank filled with water to 3 cm above the soil surface for 40 h at 21°C.

Tomato fruits (green, pink, or red; cv. Orlando) were obtained from a local market. Artificial ripening was by a combination of 16 h incubation in 50 mM LiCl, 0.5 mM indoleacetic acid, 0.1 mM benzyladenine, and 0.6 mM aminooxyacetic acid, followed by 2 h of wounding (as modified from [14]).

2.2. *Construction of a tomato cDNA library*

The construction of a tomato cDNA library from RNA induced under artificial ripening conditions, as well as filter hybridization conditions have been described previously [15].

2.3. *DNA sequence analysis*

The tomato ADH cDNA insert was subcloned as en *Eco*RI fragment in pUC18, and named TADH1. Sequences were obtained by the dideoxy chain termination method [16].

2.4. *Nucleic acid gel blotting and hybridization*

Genomic DNA was essentially prepared as described [17], followed by a CsCl gradient. DNA gels were blotted onto Hybond N^+ (Amersham) and the probe labelled by using a Megaprime kit according to the manufacturer's specifications (Amersham). Hybridization was carried out at 60°C mainly as described [18].

Total RNA was purified as reported [19], and the poly(A)$^+$ fraction prepared on oligo-dT cellulose [20]. Six percent formaldehyde gels

were blotted on Hybond N (Amersham). RNA gel blot hybridization was at 42°C in 5× SSPE/0.5% SDS/50% formamide/5% Denhardt's solution/0.1 mg/ml of denatured herring sperm DNA [15]. For RNA slot blot analysis, samples were prepared and fixed onto Hybond N+ as recommended by Amersham.

3. RESULTS

3.1. *Screening of a λgt11 tomato cDNA library and sequencing of the* Adh *clone*

Upon screening a tomato cDNA library for 1-aminocyclopropane-1-carboxylic acid (ACC) synthase [15], one positive clone was found which contained an extra fragment, identified as *Adh* after sequence analysis and comparison with sequences in the database. The fragment was subcloned in pUC18 and named TADH1. The sequence is presented in Fig. 1. The cDNA of 594 bp includes part of the coding region (1–390 bp, containing the last 130 amino acids) and possesses an untranslated sequence of 204 bp. Both the catalytic domain and coenzyme binding domain of the ADH protein reside, at least in part, in the peptide encoded by TADH1. This explains the high degree of similarity with other ADH species. On the amino acid level, TADH1 is 96% homologous to potato ADH, and 79–84% homologous to maize ADH2 and ADH1, respectively. On the DNA level these homologies are 96% between tomato and potato and 71% between tomato and maize.

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          9          18          27          36          45          54
CGT AAA CCA GTT CAA GAG GTA ATT GCT GAG ATG ACT GAT GGC GGA GTC GAT AGG
R   K   P   V   Q   E   V   I   A   E   M   T   D   G   G   V   D   R

         63          72          81          90          99         108
AGT GTG GAA TGT ACT GGT CAC ATT GAT GCT ATG ATT TCA GCA TTT GAA TGT GTC
S   V   E   C   T   G   H   I   D   A   M   I   S   A   F   E   C   V

        117         126         135         144         153         162
CAT GAT GCC TGG GGA GTG GCG GTT CTT GTT GGT GTA CCC CAT AAA GAA GCT GTG
H   D   G   W   G   V   A   V   L   V   G   V   P   H   K   E   A   V

        171         180         189         198         207         216
TTC AAG ACA CAT CCT CTG AAC TTT TTG AAT GAA CGG ACT CTC AAA GGA ACC TTC
F   K   T   H   P   L   N   F   L   N   E   R   T   L   K   G   T   F

        225         234         243         252         261         270
TTT GGA AAC TAC AAA CCT CGT TCG GAT ATT CCT TGT GTT GTT GAG AAA TAC ATG
F   G   N   Y   K   P   R   S   D   I   P   C   V   V   E   K   Y   M

        279         288         297         306         315         324
AAC AAA GAA CTT GAA TTG GAG AAA TTC ATC ACT CAT ACA CTT CCA TTT GCT GAA
N   K   E   L   E   L   E   K   F   I   T   H   T   L   P   F   A   E

        333         342         351         360         369         378
ATC AAT AAG GCT TTC GAT TTA ATG CTG AAG GGA GAA GGC CTT CGT TGC ATC ATC
I   N   K   A   F   D   L   M   L   K   G   E   G   L   R   C   I   I

        387                 403         413         423         433         443
ACC ATG GCG GAC TAA ACTTTCTGTCCTAGAAAAGGAGCTTCTACTGTTTGAGAAAAAAGACCAAT
T   M   A   D   .

       453         463         473         483         493         503         513
AAATTGTCACTGTCTTATTTTCCCTTTCGTGTTTGGTTGAGTTGTAACATTCCATCCATGTCCTCTTCTTT
       523         533         543         553         563         573         583
TGTCTTTTGCTTAGATGTTGTGCTTTGCCATATCTCTTTCGATTCTTGTAAAAAATGCAAATTCTCTCAA
       593
AAAAAAAAAAA
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Fig. 1. Nucleotide and deduced amino acid sequences of the tomato ADH cDNA TADH1 (EMBL accession number X60600).

Fig. 2. Genomic DNA gel blot analysis of tomato. Ten μg of total DNA was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hin*dIII (lane 3), and *Xba*I (lane 4). One, two, and five copy equivalents of TADH1 are in lanes 5, 6 and 7, respectively. The *Eco*RI-*Nco*I fragment of TADH1 (380 bp) was megaprime labelled and used as a probe. Exposure was for 36 h on flash-sensitized film.

3.2. *Detection of* Adh *sequences in tomato genomic DNA*

Genomic DNA gel blot analysis with the coding part of TADH1 as a probe revealed a fairly complex hybridization pattern (Fig. 2). Restriction of tomato genomic DNA with *Bam*HI, *Eco*RI, *Hin*dIII, and *Xba*I showed 3–6 hybridizing bands, all of which had intensities of one or two copy equivalents. This complexity indicated the presence of a multigene family in tomato, consistent with the three *Adh* genes found in potato, another genus of the *Solanaceae* family [8].

3.2. *Analysis of the expression of tomato ADH at the mRNA level*

The anaerobic induction of *Adh* messenger was tested in tomato seedlings and roots. As shown in Fig. 3A, a 1.4-kb mRNA was detected at low levels in uninduced

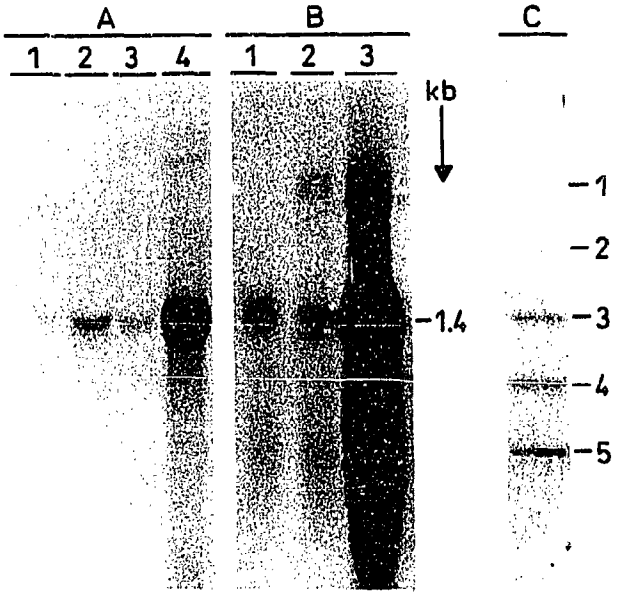

Fig. 3. Expression of tomato ADH under anaerobic conditions (A), during ripening (B and C), and after wounding (C). A ^{32}P-labelled *Eco*RI-*Nco*I fragment of TADH1 was used as a probe. Hybridization was at 42°C in a buffer containing 50% formamide. (A) RNA gel blot of submerged etiolated tomato seedlings and waterlogged tomato roots. Each lane contains 100 μg of total RNA. (Lane 1) Etiolated seedlings; (lane 2) after 20 h submergence; (lane 3) root tissue; (lane 4) root tissue after 40 h waterlogging. Exposure was 24 h on flash-sensitized film. (B) RNA blot analysis of tomato fruit at different stages. Each lane contains 10 μg of poly(A)$^+$ RNA. (Lane 1) Green tissue; (lane 2) pink tissue; (lane 3) artificially ripened pink tissue. Exposure was 24 h on flash-sensitized film. (C) RNA slot blot analysis of naturally and artificially ripened tomato fruit, or after wounding. Each lane contains 25 μg of total RNA. (1) Green tissue; (2) pink tissue; (3) red ripe tissue; (4) artificially ripened pink tissue; (5) pink tissue after 8 h wounding treatment. Exposure was 60 h on flash-sensitized film.

tissues. After 20 h of submergence of tomato seedlings, the *Adh* mRNA level was at least 10-fold increased, whereas at least 30-fold induction was observed in waterlogged tomato roots after 40 h.

Fig. 3B presents an RNA gel blot of tomato fruit at different ripening stages. Very little if any difference was seen between mRNA levels in mature green and pink tomatoes. By contrast, there was a dramatic increase of *Adh* messenger when fruits were artificially ripened. At least 50-fold induction compared to the level in green tissue was observed. This induction range is comparable to that demonstrated for ACC synthase under the same conditions [15]. From Fig. 3C it was concluded that approximately the same induction of *Adh* messenger was reached upon natural ripening. The highest induction was observed after 8 h of wounding of pink fruit tissue.

In all cases, the RNA blots were probed with a coding region fragment of TADH1 (*Eco*RI-*Nco*I fragment, 380 bp). Because *Adh* genes were found to be highly conserved among different species [4,8], and given the results of the DNA gel blot, it is very likely that the probe revealed all classes of *Adh* mRNA.

4. DISCUSSION

In this paper we report cDNA cloning and expression analysis of tomato *Adh*. The partial cDNA covers the last third of the ADH protein and is highly conserved with both monocotyledonous and dicotyledonous *Adh* genes (79–96%).

RNA gel blot analysis indicated the existence of a 1.4-kb messenger, the same size as was found for the *Arabidopsis thaliana Adh* mRNA [6]. High induction of tomato *Adh* mRNA was observed under anaerobiosis, as expected for this terminal step of glycolysis [10]. Comparable induction levels have been reported for *Adh* from several plants [2,4,6–8]. Hypoxia is not only created under conditions of waterlogging, but also occurs when fruits and vegetables are stored under controlled or modified atmosphere.

Research concerning glycolytic activities during fruit ripening and storage has so far been limited to the analysis of enzymatic activities [21–25]. Glycolytic carbon flux was reported to increase 2–3-fold during ripening [21]. Enhancement of gycolytic activity was detected both in ethylene-treated [22] and in naturally ripened fruit [23,24]. A recent paper [25] described the enhancement of ADH activity in avocado fruit ripened in air and under hypoxia. Our results on accumulation of *Adh* mRNA during tomato fruit ripening are consistent with those data. There is a low constitutive expression of *Adh* mRNA in mature green tomato fruit, and in the pink stage. The fact that the hybridization occurred to a broad rather than a discrete band may indicate the presence of mRNAs from several genes. At the protein level, new ADH isozymes have also been found in avocado late in ripening [25]. Under both natural and artificial ripening conditions, tomato *Adh* mRNA accumulated to a level at least 50-fold over green fruit. High induction under artificial ripening conditions is probably the result of the combined effects of anoxia, with auxin [4] and wounding. An 8-h wounding treatment of pink tomato fruit was also shown to result in *Adh* messenger levels comparable to those in ripening fruit. Recently, potato *Adh* was shown to be responsive to different stresses [8]. Further research will be needed to investigate possible effects of different inducing factors of *Adh* during the ripening process. This is of particular importance with respect to the maintenance of flavour of fruits during post-harvest periods.

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REFERENCES

[1] Eklund, H., Nordström, B., Zeppenzauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., Söderberg, B.O., Tapia, O. and Bränden, C.I. (1976) J. Mol. Biol. 102, 27–59.

[2] Dennis, E.S., Sachs, M.M., Gerlach, W.L., Finnegan, E.J. and Peacock, W.J. (1985) Nucleic Acids Res. 13, 727–743.

[3] Trick, M., Dennis, E.S., Edwards, K.J.R. and Peacock, W.J. (1988) Plant Mol. Biol. 11, 147–160.

[4] Xie, Y. and Wu, R. (1989) Plant Mol. Biol. 13, 53–68.

[5] Xie, Y. and Wu, R. (1990) Gene 87, 185–191.

[6] Chang, C. and Meyerowitz, E.M. (1986) Proc. Natl. Acad. Sci. USA 83, 1408–1412.

[7] Llewellyn, D.J., Finnegan, E.J., Ellis, J.G., Dennis, E.S. and Peacock, W.J. (1987) J. Mol. Biol. 195, 115–123.

[8] Matton, D.P., Constabel, P. and Brisson, N. (1990) Plant Mol. Biol. 14, 775–783.

[9] Sachs, M.M., Freeling, M. and Okimoto, R. (1980) Cell 20, 761–767.

[10] Dennis, E.S., Walker, J.C., Llewellyn, D.J., Ellis, J.G., Singh, K., Tokuhisa, J.G., Wolstenholme, D.R. and Peacock, W.J. (1987) in: Plant Molecular Biology (von Wettstein, D. and Chua, N.-H. eds.) pp. 407–417, Plenum Press, New York.

[11] Olive, M.R., Walker, J.C., Singh, K., Dennis, E.S. and Peacock, W.J. (1990) Plant Mol. Biol. 15, 593–604.

[12] Christie, P.J., Hahn, M. and Walbot, V. (1991) Plant Physiol. 95, 699–706.

[13] Springer, B., Werr, W., Starlinger, P., Bennett, D.C., Zokolica, M. and Freeling, M. (1986) Mol. Gen. Genet. 205, 461–468.

[14] Sato, T. and Theologis, A. (1989) Proc. Natl. Acad. Sci. USA 86, 6621–6625.

[15] Van Der Straeten, D., Van Wiemeersch, L., Goodman, H.M. and Van Montagu, M. (1990) Proc. Natl. Acad. Sci. USA 87, 4859–4863.

[16] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.

[17] Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983) Plant Mol. Biol. Reporter 1, 19–21.

[18] Church, G.M. and Gilbert, W. (1984) Proc. Natl. Acad. Sci. USA 81, 1991–1995.

[19] Rodrigues-Pousada, R., Van Montagu, M. and Van Der Straeten, D. (1990) Technique 2, 292–294.

[20] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[21] Solomos, T. and Laties, G.G. (1974) Plant Physiol. 54, 506–511.

[22] Stitt, M., Cséke, C. and Buchanan, B. (1986) Plant Physiol. 80, 246–248.

[23] Bennett, A.B., Smith, G.M. and Nichols, B.G. (1987) Plant Physiol. 83, 973–976.

[24] Ball, K.L., Green, J.H. and Rees, T. (1991) Eur. J. Biochem. 197, 265–269.

[25] Kanellis, A.K., Solomos, T. and Roubelakis-Angelakis, K.A. (1991) Plant Physiol. 96, 269–274.