A Nuclear Gene Encoding Mitochondrial Proline Dehydrogenase, an Enzyme Involved in Proline Metabolism, Is Upregulated by Proline but Downregulated by Dehydration in Arabidopsis

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Proline is one of the most common compatible osmolytes in water-stressed plants. The accumulation of proline in dehydrated plants is caused both by the activation of proline biosynthesis and by the inactivation of proline degradation; a decrease in the level of accumulated proline in rehydrated plants is caused both by the inhibition of proline biosynthesis and by the activation of proline degradation. The proline biosynthetic pathway has been well characterized, but the degradation of proline is poorly understood. Sequence analysis of an Arabidopsis cDNA clone, ERD5 (for early responsive to dehydration stress), isolated from plants dehydrated for 1 hr, revealed that it encodes a protein with identity to products of the yeast PUT1 (for proline utilization) gene (23.6% over 364 amino acids) and the Drosophila sluggish-A gene (34.5% over 255 amino acids). Their gene products are precursors of proline oxidases (dehydrogenase) (EC 1.5.99.8), which are the first enzymes involved in the conversion of proline to glutamic acid. Proline oxidase is localized in mitochondria. RNA gel blot analysis demonstrated that transcripts of the ERD5 gene were undetectable when plants had been dehydrated for 10 hr, but large amounts of the transcript accumulated when plants subsequently were rehydrated. Elevated levels of the transcript were also found in plants that had been incubated in a medium that contained proline. Immunologically, we showed that the product of ERD5 is localized in the mitochondrial fraction and accumulates in response to proline in cultured cells. Fusion genes for ERD5 and PUT1 complemented a put1 mutant of yeast, allowing put1 to grow with proline as the source of nitrogen. These results suggest that ERD5 encodes a precursor of proline dehydrogenase (oxidase), which is regulated at the level of mRNA accumulation in both dehydrated and rehydrated plants.

INTRODUCTION

Many plants accumulate compatible osmolytes, such as sugar alcohols, proline, or glycine betaine, when they are subjected to drought or salinity stress (Hellebust, 1976; Delauney and Verma, 1993). These stresses are the major factors that limit plant growth and crop productivity (Boyer, 1982), and compatible osmolytes appear to protect plants from such stresses. There are many reports that indicate a positive correlation between the accumulation of proline and adaptation to osmotic stress in plants (see Delauney and Verma, 1993). Moreover, transgenic tobacco plants genetically engineered to accumulate mannitol or proline are able to tolerate higher salinity better than control tobacco plants do (Tarczynski et al., 1993; Kavi Kishor et al., 1995).

Among the compatible organic solutes, proline is probably the most widely distributed osmolyte; it accumulates in eubacteria, protozoa, and marine invertebrates (McCue and Hanson, 1990; Delauney and Verma, 1993). The biosynthetic pathway to proline in plants has been well characterized by functional complementation of mutant strains of Escherichia coli (Delauney and Verma, 1990; Hu et al., 1992; Delauney et al., 1993). In plants, L-proline is produced from L-glutamic acid via Δ^1-pyrroline-5-carboxylate (P5C). Production is catalyzed by two enzymes, P5C synthetase (P5CS) and P5C reductase (P5CR); production of L-proline from L-ornithine via P5C is catalyzed by ornithine-d-aminotransferase (δ-OAT) and P5CR.

Because salt stress induces the expression of the gene for P5CS and depresses it for δ-OAT in Vigna aconitifolia, the former pathway for proline synthesis has been postulated to predominate in plants under osmotic stress (Delauney and

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Verma, 1993; Delauney et al., 1993). In Arabidopsis, the expression of the gene for P5CS is strongly induced by dehydration, salt stress, and the application of abscisic acid, but the expression of the gene for P5CR is not (Yoshiba et al., 1995). Verma and co-workers have demonstrated that transgenic tobacco plants expressing soybean P5CR had an elevated level of P5CR activity but did not accumulate proline at significant levels (Szoke et al., 1992). In contrast, transgenic tobacco plants expressing moth bean P5CS produced a high level of the enzyme and subsequently accumulated 10-fold more proline than did control plants (Szoke et al., 1992; Kavi Kishor et al., 1995). Therefore, P5CS is postulated to be the rate-limiting enzyme in the biosynthesis of proline in plants.

The other important factor that controls levels of proline in plants is degradation. L-Proline is oxidized to P5C in plant mitochondria by proline dehydrogenase (oxidase) (EC 1.5.99.8), and P5C is converted to glutamate by P5C dehydrogenase (Boggess et al., 1975; Elthon and Stewart, 1981). This oxidation of proline is inhibited during the accumulation of proline under water stress and is activated in rewater-stressed plants (Stewart et al., 1977; Rayapati and Stewart, 1991). The isolation of the gene for proline oxidase would be a valuable contribution to efforts to understand the role of proline dehydrogenase in the accumulation of proline in plants. Unfortunately, it has proven difficult to purify and isolate the enzyme in substantial amounts, because the activity is bound to the matrix side of the inner membrane of mitochondria and the enzyme seems to donate electrons directly to the respiratory electron transport system (Elthon and Stewart, 1981, 1982). Rayapati and Stewart (1991) succeeded in solubilizing proline dehydrogenase activity from plant mitochondria. However, proline dehydrogenase in plants remains to be characterized molecularly.

We are investigating plant responses to water stress, and we have isolated 16 groups of cDNAs (ERD clones, early responsive to dehydration clones) that correspond to genes expressed in Arabidopsis after dehydration for 1 hr. A differential screening technique (Kiyosue et al., 1994a) was used. Sequence analysis of ERD clones revealed that ERD1 is homologous with the cDNA for the regulatory subunit of the Clp ATP-dependent protease in E. coli, and its predicted product contains a putative chloroplast-targeting signal at the N terminus; ERD10 and ERD14 are similar to cDNAs for group II late embryogenesis abundant (LEA) proteins; ERD11 and ERD13 are homologous with cDNAs for glutathione S-transferases (GST); and ERD2, ERD8, and ERD16 are identical to cDNAs for heat shock proteins HSP70-1 and HSP81-2 and the ubiquitin extension protein, respectively (Kiyosue et al., 1993a, 1993b, 1994a, 1994b).

In this report, we characterize one of the remaining ERD clones, ERD5, as a cDNA for a proline dehydrogenase based on its sequence homology with proline oxidases of yeast and Drosophila, the negative correlation between its pattern of gene expression and the proline content of Arabidopsis plants under water-stressed conditions, the subcellular localization of its products, and the functional complementation of the put1

(for proline utilization) mutation in yeast. We show that a gene corresponding to ERD5 is repressed under dehydration conditions but is strongly induced by rehydration and the exogenous application of proline. The induced accumulation of the ERD5 mRNA in rehydrated plants suggests that proline dehydrogenase might play a crucial role in the degradation of proline in plants after the termination of water stress.

RESULTS

Sequence Analysis of the ERD5 cDNA

The nucleotide sequence of insert in the ERD5 clone was determined. The cDNA had been isolated by differential hybridization from a cDNA library of Arabidopsis plants that had been dehydrated for 1 hr (Kiyosue et al., 1994a). The 1852-bp insert contained one long open reading frame encoding 499 amino acid residues (calculated molecular mass of 55.04
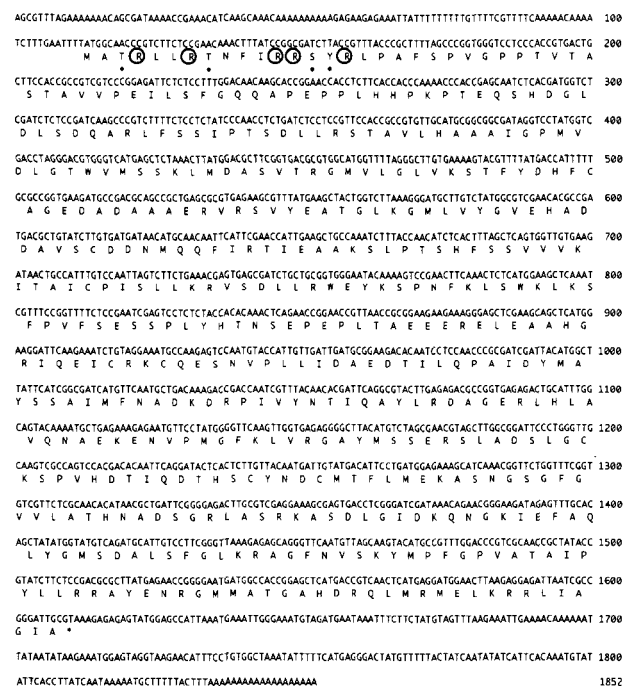

Figure 1. The Nucleotide and Deduced Amino Acid Sequence of the ERD5 cDNA.

The nucleotide sequence of the cDNA clone ERD5 (insert 2; Kiyosue et al., 1994a) includes a putative coding region and 5' and 3' noncoding regions. The amino acid sequence of the putative coding region is shown beneath the nucleotide sequence. Positively charged amino acids and hydroxylated amino acids at the N terminus are indicated by circles and dots, respectively. An asterisk indicates the in-frame stop codon. The ERD5 sequence has been submitted to the DDBJ data base as accession number D83025.

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At  ERD5    MA----TRLL RT--NFI--- ---RRSYRLP -----AF--- ---------- ---------- ---------- SPVGPPTVTA STAVVPEILS FGQQAPEPPL   50
Sc  PUT1    MI----AS-- KS--SLL--- ---VTKSRIP -----SL--- ---------- ---------- ---------- C---FPLI-- KRSYVSKTPT HSNTAANLMV   43
Dm  PUT1    MALLRSLSAQ RTAISLVYGR NSSKSSNSVA VAACRSFHQR GNGSTSIAGE GAASESTRGV NGARFLHSGD RPLQASTLVQ PEVVSSETVK RSMKQESSQE  100

Consensus   MA-----S.. RT--SL.--- ---..S.R.P -----SF--- ---------- ---------- ---------- .P...PT... ...VVSET.. .S..A.....  100


At  ERD5    HHPKPTEQSH -DGLDLS--D QARLFSSIPT SDLLRSTAV- L---HAAAIG PMVDLGTW-- -VMS----SK LMDASV---- ----TRGMVL -------GL-  120
Sc  PUT1    ETPAANPNGN -SVMAPP--N SINFLQTLPK KELFQ----- L-----GFIG -IATLNSF-- -FLN----TI IKLFPY---- ----IPIPVI -------KF-  106
Dm  PUT1    KNPSPAGSPQ RDPLDVSFND PIAAFKSKTT GELRAYLVY MICSSENLVE HNMTLMKWSK NVLGQRLFTL LMKATFYGHF VAGEDQIKII PTLERLRSFG  200

Consensus   ..PLP..... -D.LD.S--D .I..F.S.PT .ELR....V- L---....IG ...TL.W-- -VL.----T. LM.A..---- ----.I.VI -------.F-  200


At  ERD5    VKSTF-Y--- DHFCAGEDAD AAAE-RVRSV YEATGLKGML VYGVEH--AD ---------- ----DAVSCD DNMQQFIRTI EAA------K SLPTS-----  188
Sc  PUT1    FVSSL-M--- ---CCGENFK EVIE-CGKRL QKRGISNMML SLTIEN--SE ---------- ----GTKSLS STPVDQI-VK ETI------S SVHNI-----  170
Dm  PUT1    VKPILDYSVE EDITQEEABK REVEBSVSSA GDKKEEGSMP QYHVDKSFAD RRYKVSSART YFYLNEATCE RNMEIFIKCL EAVSGATFGT GITAIKLTAL  300

Consensus   VKS.L-M--- ...C.GE.K .E-.V.S. ........ML .Y.VE.--AD ---------- ----...SC. .NM..FI... EA.------. S...I-----  300


At  ERD5    -------HFS SVVVK----- ---------- IT-AICPISL LKRVSDL--- -LRWEYKS-- --PNFKLSWK LKSFPVFSES SPLYHTNSEP EPLTAEEERE  257
Sc  PUT1    -------LLP NIIGQ----- ---------- LE-SKPITDI APGYIAL--- -KPSALVD-- --NPHEVLYN FSNPAYKAQR DQLIENCSKI TKEIFELNQS  239
Dm  PUT1    GRPQLLLQLS EVIMRTRKYM EDMVGGQGNV LTHHKTIKDL EKYYATLGDN KDVKEFLNNV TSDKEGILHL FPWSGIVDED SQLSDTFRVP DPQTGQMRRL  400

Consensus   -------.LS .VI..----- ---------- LT-.K.I.DL .K.Y..L--- -...E..--- --.....L.. F........E. SQL..T.S.P .P.T.E..R.  400


At  ERD5    LEAAHGRIQE IC-----RKC QESNVPLLID AEDTILQPAI DYM----AYS SAIMFNADKD RPIVYNIIDA VLRDAGERLH LAVQNAEKEN VPMGHKLVRG  348
Sc  PUT1    LLKKYPERKA PF-----MVS TIDAEKYDLQ ENGVYELQRI LFQ----KFN PT----SSKL ISQV-GTMQL VLRDSGDHIL HELKLADENG YKLGLKLVRG  325
Dm  PUT1    ISQIPPKEEE MFRNMIRRLN TIVKAAADLD VRIMVDAEQT YFQPAISRIT LEMMRKYNKD KAIVFNTVDC VLRETFREVN TDLEQAKRQN FYFGAKLVRG  500

Consensus   L....P...E .F-----R.. TI.....DLD .........I .FQ----... ...M....KD ..IV.NT.D. VLRD.G.... ...L..A..N ...G.KLVRG  500


At  ERD5    AYMSSERSLA DSLGCKSPVH DTIQDTHSCY N----DCM-- -TFLMEKASN GSGFGVVLAT HNADSGRLAS RKASDLGIDK QNGKIEFADL YGMSDALSFG  441
Sc  PUT1    AYIHSEKNRN QIIFGDKTGT DENYD-RIIT Q----VVN-- -DLIINGEDS YFGHLVVASH NYQSQMLVTN LLKSTQDNSY AKSNIVLGDL LGMADNVTYD  417
Dm  PUT1    AYMDQERDRA KSLGYPDPVN PTFEATTDMY HRTLSECLRR IKLMKDCDDD ARKIGIMVAS HNEDTVRFAI QQMKEIGISP EDKVICFGDL LGMSDYITFP  600

Consensus   AYM.SER.RA .SLG...PV. DT..DT...Y .----.C.-- -.L.....D. ...G.GVV.A. HN.D..R.A. ...S..GIS. ....I.FGDL LGM.D..TF.  600


At  ERD5    LL--KRAGFNV SKYMPHGPVA TAIPYLLRRA YENRGMMATG AHDRQLMRME LKRRLIAGIA ---------- -   499
Sc  PUT1    LITNHGAKNI IKYVPNGPPL ETKDYLLRRF LQENSDAVRS DNGWFLIKAI AKSIPKRVGL ---------- -   476
Dm  PUT1    LL--GQAGYSA YKYIPVGPVE EVLPYLSRRA QENKGVLKKI KKEKRLLLSE IRRRLMRGQL FYKPKGNYVP I   669

Consensus   LL--..AG.N. .KYIPLGPV. E..PYLLRRA .EN.G.... ....L..E .KRRL.RG.L ---------- -   671
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Figure 2. Comparison of Deduced Amino Acid Sequences.

A comparison is shown of the deduced amino acid sequences of the ERD5 protein of Arabidopsis (At) and the products of the yeast (Sc) *PUT1* gene (Wang and Brandriss, 1987) and the Drosophila sluggish-A gene (Dm PUT1; Hayward et al., 1993). When at least two of the three sequences had the same residue at a given position, this residue was included in the consensus sequence. Dashes indicate gaps introduced to maximize homology. Amino acid residues common to all three sequences are indicated by boxes. Nonconserved amino acid residues are indicated by dots.

kD; Figure 1). A putative polyadenylation signal, AATAAA (Fitzgerald and Shenk, 1981), was found in two places: at positions 1657 to 1662 and 1813 to 1818. The N terminus of the putative ERD5 protein had features characteristic of proteins that are imported into mitochondria. The features are as follows: the first 16 residues included five positively charged amino acids, no negatively charged residues, and four hydroxylated amino acids. Comparison of the deduced amino acid sequence with other sequences in the standard data bases revealed that the ERD5 protein was 34.5% (>255 amino acids) and 23.6% (>364 amino acids) identical to the precursors of proline oxidase (EC 1.5.99.8) of Drosophila (product of the sluggish-A gene; Hayward et al., 1993) and *Saccharomyces cerevisiae* (product of the *PUT1* gene; Wang and Brandriss, 1987), respectively. Figure 2 compares the deduced amino acid sequences of the three proteins. The greatest similarity among these sequences was found in two regions, between residues 307 and 354 and between residues 426 and 485.

Genomic DNA Gel Blotting

Arabidopsis genomic DNA was digested with five restriction enzymes, and after electrophoresis and blotting, fragments were hybridized to the ^{32}P-labeled ERD5 cDNA. Under high-stringency conditions, the ERD5 cDNA hybridized with one fragment in each case (data not shown). Because an XbaI fragment of genomic DNA corresponding to the hybridized XbaI band was shown to contain one ERD5 sequence, which was interrupted with introns (T. Kiyosue and K. Shinozaki, unpublished results), each of these hybridized bands seemed to contain one gene corresponding to ERD5. Under low-stringency hybridization conditions, additional weak bands were detected (data not shown), indicating the presence of an *ERD5*-related gene(s) in the Arabidopsis genome.

Effects of Dehydration, Rehydration, and Heat and Cold Treatments on Expression of the *ERD5* Gene, and Relationships between the Accumulation of Proline and the Expression of Genes Encoding ERD5 and P5CS

To investigate the effects of environmental stress on the expression of the *ERD5* gene, RNAs extracted from whole 4-week-old plants that had been grown in pots and subjected to dehydration, rehydration, or heat or cold treatments were
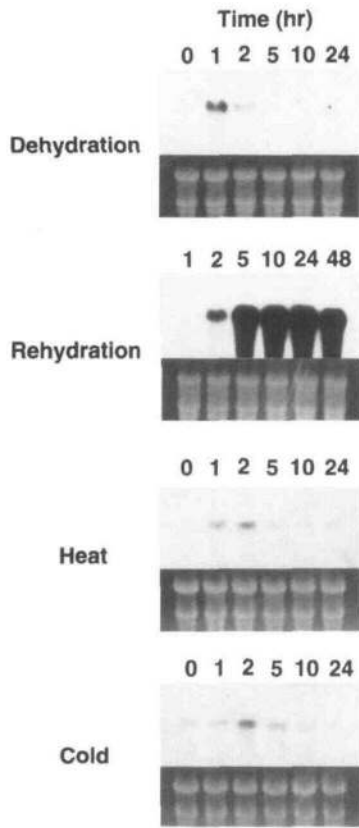
Time (hr)

Figure 3. Expression of the *ERD5* Gene in Response to Various Types of Stress.

Shown is an RNA gel blot analysis of *ERD5* transcripts in Arabidopsis plants subjected to dehydration, rehydration, or heat or cold stress. Ten micrograms of total RNA extracted from 1-month-old Arabidopsis plants was loaded in each lane. Samples were prepared from plants that had been treated by dehydration (Dehydration) or by dehydration for 10 hr and subsequent rehydration for 1 to 48 hr (Rehydration) or had been incubated at 40°C (Heat) or 4°C (Cold). The numbers above each lane indicate the number of hours after the initiation of the specified treatment before the isolation of RNA. Total RNAs were fractionated on 1% agarose gels that contained formaldehyde, visualized by staining with ethidium bromide (gels shown below), and transferred to nylon membranes. Filters were hybridized with a ^{32}P-labeled fragment of ERD5 cDNA at 42°C and washed in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS at 60°C. The fragments of cDNA that hybridized to specific RNAs were visualized by autoradiography (gels shown above).

examined by gel blotting (Figure 3). The level of *ERD5* mRNA increased transiently within 1 hr of the start of dehydration and then decreased. No *ERD5* mRNA was detected after dehydration for 5 hr (Figures 3 and 4A). During this treatment, the level of the mRNA for P5CS, the enzyme that catalyzes the first step in the biosynthesis of proline from glutamic acid, increased; it reached a maximum within 5 hr of the beginning of dehydra-

tion, with the subsequent accumulation of proline (Figure 4A). In contrast, when plants were dehydrated for 10 hr and then rehydrated by transfer to water, the level of *ERD5* mRNA began to increase within 2 hr, and large amounts of the mRNA were detected after rehydration for 5 to 48 hr (Figures 3 and 4B). During this treatment, the level of the transcript of the gene encoding P5CS, which had been elevated by dehydration, began to decrease within 1 hr and reached a minimum within 10 hr after the beginning of rehydration. Proline content decreased rapidly for 10 hr from the beginning of rehydration and then gradually for the next 38 hr (Figure 4B). The transient increase in the level of the mRNA transcribed from the *ERD5* gene, which was seen in briefly dehydrated plants, was also detected in briefly heat- and cold-stressed plants, but no strong induction by heat or cold stress was observed (Figure 3).
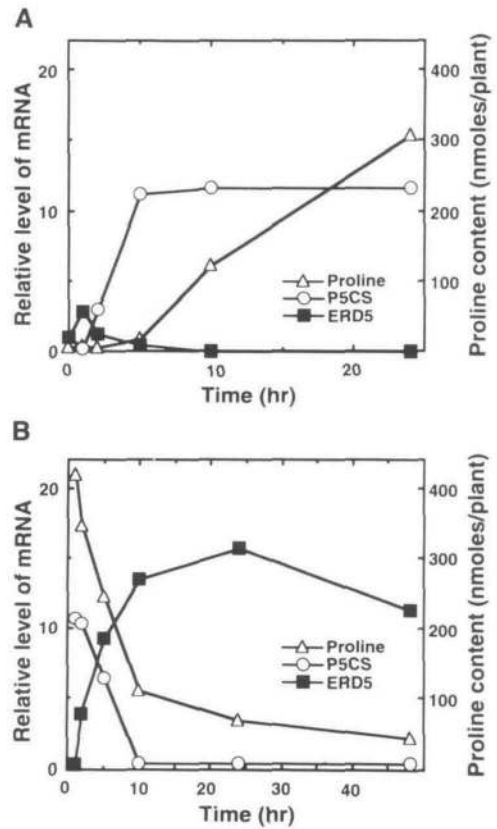

Figure 4. The Relationships between the Accumulation of Proline and the Level of Expression of the Gene for P5CS and That of the *ERD5* Gene during Dehydration and Rehydration.

The radioactivity retained on the nylon filters in Figure 3 was quantified with a BioImage Analyzer (Fuji Film Co., Tokyo, Japan) and plotted as shown. The details of the accumulation of P5CS transcripts and of proline are replotted from data that we published previously (Yoshiba et al., 1995).

(A) Dehydration of Arabidopsis plants.

(B) Rehydration of 10-hr dehydrated Arabidopsis plants.

A

B
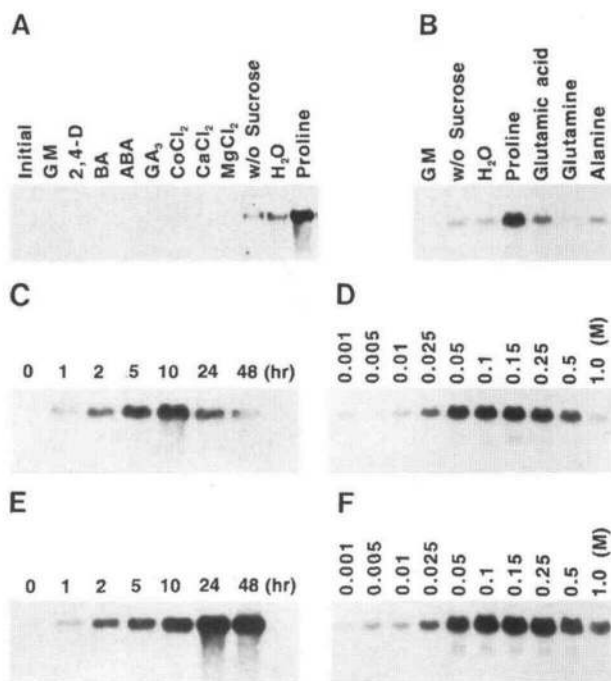

C

D

E

F

Figure 5. Expression of the *ERD5* Gene in Rosette Plants in Response to Exogenous Proline.

(A) Expression of the *ERD5* gene in rosette plants in response to various compounds. One-month-old axenic Arabidopsis plants (Initial) were transferred to GM liquid medium (GM) with 10^{-4} M 2,4-D (2,4-D) 6-benzylaminopurine (BA), abscisic acid (ABA), gibberellic acid (GA$_3$), cobalt chloride (CoCl$_2$), calcium chloride (CaCl$_2$), magnesium chloride (MgCl$_2$), without sucrose (w/o Sucrose), or with 0.26 M L-proline instead of sucrose (Proline), or to deionized water (H$_2$O), and were incubated for 10 hr.

(B) Expression of the *ERD5* gene in rosette plants in response to several L-amino acids. One-month-old axenic Arabidopsis plants were transferred to GM liquid medium with 0.26 M proline, glutamic acid, glutamine, or alanine instead of sucrose and incubated for 10 hr.

(C) Time course of expression of the *ERD5* gene in rosette plants in response to exogenous L-proline. One-month-old axenic Arabidopsis plants were transferred to GM liquid medium with 0.26 M L-proline instead of sucrose and incubated for 1, 2, 5, 10, 24, or 48 hr.

(D) Dose–response analysis of expression of the *ERD5* gene in rosette plants in response to exogenous L-proline. One-month-old axenic Arabidopsis plants were transferred to GM liquid medium with 0.001, 0.005, 0.01, 0.025, 0.05, 0.1, 0.15, 0.25, 0.5, or 1.0 M L-proline instead of sucrose and incubated for 10 hr.

(E) Time course of expression of the *ERD5* gene in rosette plants in response to exogenous D-proline. One-month-old axenic Arabidopsis plants were transferred to GM liquid medium with 0.26 M D-proline instead of sucrose and incubated for 1, 2, 5, 10, 24, or 48 hr.

(F) Dose–response analysis of expression of the *ERD5* gene in rosette plants in response to exogenous D-proline. One-month-old axenic Arabidopsis plants were transferred to GM liquid medium with 0.001, 0.005, 0.01, 0.025, 0.05, 0.1, 0.15, 0.25, 0.5, or 1.0 M D-proline instead of sucrose and incubated for 10 hr.

Total RNA was isolated, fractionated on 1% agarose gels that contained formaldehyde, visualized by staining with ethidium bromide (data not

Induction of the *ERD5* Gene by Exogenous Application of Proline

RNA gel blot analysis was also used to examine the effects of several compounds on expression of *ERD5* (Figures 5A and 5B). No expression of *ERD5* was observed when 1-month-old rosette plants of Arabidopsis, which had been grown on GM agar medium (Valvekens et al., 1988), were axenically incubated in a small amount of GM liquid medium that contained any one of four plant growth regulators, namely, 2,4-D, N^6-benzyladenine, abscisic acid, or gibberellic acid; or any one of three metal chlorides, namely, CoCl$_2$, CaCl$_2$, or MgCl$_2$, at concentrations of 10^{-4} M; or any one of four amino acids, namely, L-proline, L-glutamic acid, L-glutamine, or L-alanine. In this experiment, roots were submerged in the liquid medium, but aerial parts were not. When the plants were incubated in GM liquid medium without sucrose or in deionized water, a slight induction in expression of the *ERD5* gene was detected. Strong induction of the gene was observed when plants were incubated in GM liquid medium that contained 0.26 M L-proline. The time course of this induction of the *ERD5* gene by L-proline was determined (Figure 5C). The accumulation of the *ERD5* mRNA began within 1 hr after the transfer of the plants to GM liquid medium that contained 0.26 M L-proline, reached a maximum after 10 hr of incubation, and then decreased.

Dose–response analysis revealed that incubation in 0.001 to 1.0 M L-proline for 10 hr induced expression of the *ERD5* gene in treated plants (Figure 5D). The induction of expression of the *ERD5* gene was also detected when D-proline was used instead of L-proline. In this case, the induction also began within 1 hr after the transfer, but the mRNA accumulated gradually and reached a maximum level within 24 hr of incubation. This elevated level was maintained for an additional 24 hr (Figure 5E). Dose–response analysis revealed that incubation of plants in 0.001 to 1.0 M D-proline for 24 hr induced expression of the *ERD5* gene (Figure 5F). Thus, D-proline induced more stable expression of *ERD5* than did L-proline in Arabidopsis plants. This stability of the induction of this gene by D-proline was also observed in cultured cells (data not shown).

The organ-specific expression of the *ERD5* gene in rosette plants that had been treated with proline was examined. One-month-old plants were incubated for 24 hr in 0.1 M D-proline and cut into three parts (leaves, roots, and the remainder). The parts were then subjected to RNA gel blot analysis. Elevated levels of *ERD5* mRNA were observed at approximately the same level in all three parts (data not shown). The *ERD5* gene

shown), and transferred to nylon membranes. Filters were hybridized with a ^{32}P-labeled fragment of the ERD5 cDNA at 42°C and washed in 0.1 × SSC, 0.1% SDS at 60°C. The fragments of cDNA that hybridized with specific RNAs are shown. Ten micrograms of total RNA was loaded in each lane.

seemed to be expressed where the mitochondria were located (Figure 6).

Immunological Detection of the ERD5 Protein

To analyze the subcellular localization of ERD5, antiserum was raised against a synthetic oligopeptide, AHDRQLMR-MELKRRL (residues 480 to 494 of the deduced ERD5 protein). This antiserum recognized a recombinant fusion protein composed of GST and the ERD5 protein that had been produced in E. coli cells, but it did not recognize the recombinant GST protein (Figure 7A). Because the predicted product of the ERD5 gene contains a putative signal for targeting to mitochondria

at its N terminus, a pellet obtained from extracts of cultured Arabidopsis cells that had been centrifuged at 12,000g was examined immunologically. Fractionation of this pellet in a Percoll density gradient clearly separated a mitochondrial fraction from a chloroplast fraction (Figure 7B). No proteins in the chloroplast fraction were recognized, whereas a 50- and a 48-kD protein were detected in the mitochondrial fraction with the specific antiserum (Figure 7C). The strong band of the 50-kD protein might correspond to a precursor form, and the weak band of the 48-kD protein might correspond to the mature form of the ERD5 protein. Alternatively, the former might correspond to a mature form and the latter might correspond to a degraded form of the ERD5 protein. The levels of these polypeptides increased within 1 day after exposure to 0.1 M D-proline, and
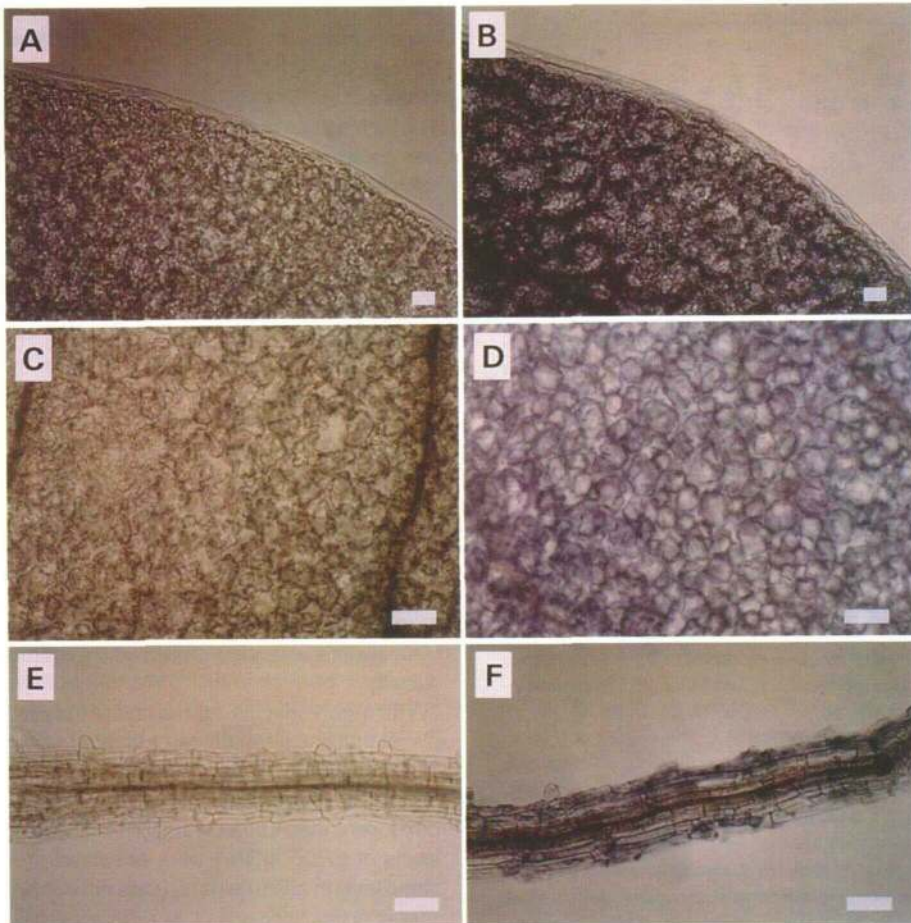
Figure 6. In Situ Analysis of Expression of the ERD5 Gene in Arabidopsis Plants.

Seven-day-old axenic seedlings that had been incubated for 24 hr in Murashige and Skoog (1962) liquid medium plus 0.1 M D-proline instead of sucrose were allowed to hybridize with single-stranded digoxigenin-labeled sense ([A], [C], and [E]) or antisense ([B], [D], and [F]) RNA probes that corresponded to ERD5.
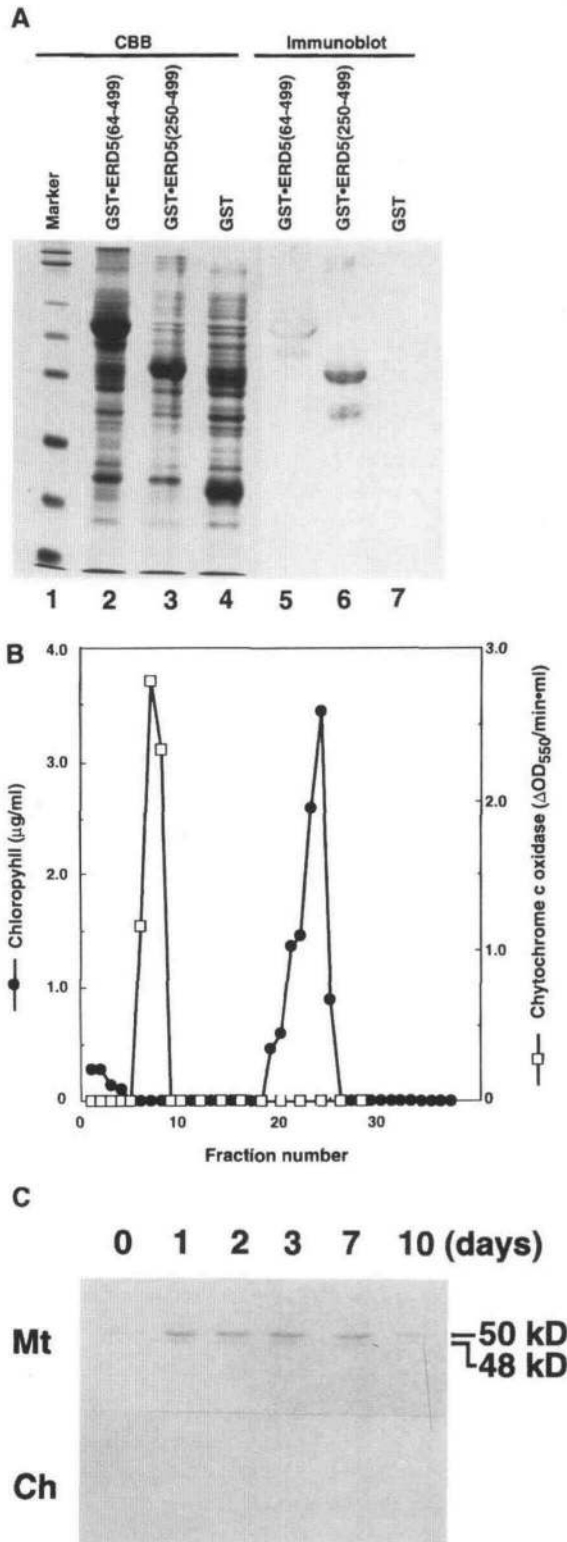(A) to (D) Leaves.
(E) and (F) Roots.
Bars = 50 mm.

this elevated level was maintained for an additional 6 days. No higher level of accumulation of the antigens was detected after a 10-day incubation. At this time, many green cells turned yellowish and seemed to be dead. Therefore, the *ERD5* gene product was deduced to be a mitochodrial protein, with its accumulation being induced by proline.

Functional Complementation of the *put1* Mutation in Yeast by ERD5

Complementation tests were conducted to determine whether the ERD5 protein could functionally substitute for the putative yeast homolog. The *put1* mutant of strain C15-1A of *S. cerevisiae* (Wang and Brandriss, 1986) was transformed with the yeast expression vector pNV7 (a 2 mm-based, high-copy-number plasmid with a *GAL1-10* promoter) that contained the *ERD5* cDNA in the sense or antisense orientation or with the unmodified vector as the control. The transformants were incubated on minimal medium supplemented with 0.5% galactose as the source of carbon, 0.1% L-proline as the inducer of expression of the introduced gene, and 0.004% L-tryptophan without ammonium sulfate. On this medium, the *put1* mutant strain grows slowly, whereas the wild-type strain grows well, because wild-type cells can use proline as the source of nitrogen but the mutant cannot (Brandriss and Magasanik, 1979). Unfortunately, none of the tested transformants grew well (data not shown). Because expression of some plant proteins in yeast cells is known to result in localization of the proteins that is different

(A) *Immunological detection of the ERD5 recombinant proteins. Total cell extracts prepared from isopropyl β-D-thiogalactoside–treated E. coli cells harboring pGST·ERD5(64-499) (lanes 2 and 5), pGST·ERD5(250-499) (lanes 3 and 6), and pGEX2T (GST; lanes 4 and 7) were analyzed by SDS-PAGE (on a 10% acrylamide gel) (lanes 2 to 4) and then immunoblotted (lanes 5 to 7) with rabbit antiserum raised against a synthetic oligopeptide (residues 480 to 494 of the deduced ERD5 protein). Marker proteins were also subjected to SDS-PAGE (lane 1). Polypeptides separated on an acrylamide gel were visualized by staining with Coomassie blue (CBB) (lanes 1 to 4).*

(B) *Distribution of chlorophyll and cytochrome c oxidase in homogenates of Arabidopsis cultured cells after Percoll discontinuous density gradient centrifugation. Experimental details are given in the text. An aliquot of each 1-mL fraction that had been collected sequentially from the bottom of the gradient was assayed for markers of specific organelles.*

(C) *Detection of the ERD5 protein in mitochondrial fractions of cultured cells. Cultured Arabidopsis cells were incubated in JPL medium with 0.1 M D-proline for 0, 1, 2, 3, 7, or 10 days. Mitochondria (Mt) and chloroplast (Ch) fractions were isolated by discontinuous Percoll density gradient centrifugation as described in the text. Protein equivalent to 5 mg of organelle fraction was loaded in each well, subjected to SDS-PAGE, and then immunoblotted with the antiserum raised against the oligopeptide derived from the deduced sequence of the ERD5 protein. Molecular mass (kD) of two detected proteins is shown at right.*

Figure 7. Expression and Immunological Detection of the ERD5 Recombinant Proteins.

from the localization in plant cells (Schaller et al., 1995), this failure of complementation might have been a result of the inappropriate localization of the ERD5 protein in yeast cells. Therefore, we constructed three fusion genes and three deletion genes by polymerase chain reaction (PCR). These genes had both the promoter and the terminator regions of the *PUT1* gene and a chimeric structural gene constructed from *PUT1* and *ERD5* coding regions or a shortened structural gene derived from the coding region of the *PUT1* gene (Figure 8A). Because these genes were created by PCR, extra amino acids were formed after PUT1 regions were inserted between PUT1 and ERD5 regions, as indicated in Figure 8A.

The plasmids containing these artificial genes, the vector control (YCp50; Parent et al., 1985), and a positive plasmid control with an intact *PUT1* gene (pWB8; Wang and Brandriss, 1986) were introduced into the C15-1A cells. Ura$^+$ transformants grown on minimal medium without uracil were streaked on minimal medium that contained 0.5% L-proline, 0.004%

L-tryptophan, and 2% glucose but no ammonium sulfate. As shown in Figure 8B, functional complementation was observed for all transformants that expressed the *PUT1–ERD5* fusion genes but not for those with the control vector or a deleted *PUT1* structural gene. Although the complementation was somewhat weak, the appearance of transformants of the *put1* mutant that expressed the fusion genes was indistinguishable from that of transformants that expressed the intact *PUT1* gene in pWB8. By contrast, many abnormal cells were observed in the case of transformants with the control vector or a deleted *PUT1* structural gene when grown on complementation test medium (data not shown).

Levels of proline oxidase were measured under noninducing conditions (ammonia as the sole source of nitrogen) and inducing conditions (ammonia and proline as sources of nitrogen) in C15-1A strains that each carried one of the plasmids listed in Figure 8A (see also Table 1). The induction of proline oxidase activity in C15-1A strains that harbored pWB8, pPUT1(1-29)·R·ERD(38-499), pPUT1(1-291)·R·ERD5(316-499), or pPUT1 (1-334)·KN·ERD5(380-499) was observed when the cells were grown in medium that contained ammonia and proline. Among the mutant yeast cells that carried these three chimeric gene constructs, the cells carrying pPUT1(1-291)·R·ERD5(316-499) grew best in liquid medium under inducing conditions (data not shown) and had the highest proline oxidase activity. In contrast, the low proline oxidase activity of the mutant *put1* strain did not increase when induced in strains with the plasmid that lacked the fusion gene or the intact *PUT1* gene. These results indicate that the products of the *PUT1–ERD5* fusion genes function as proline oxidases in yeast cells.
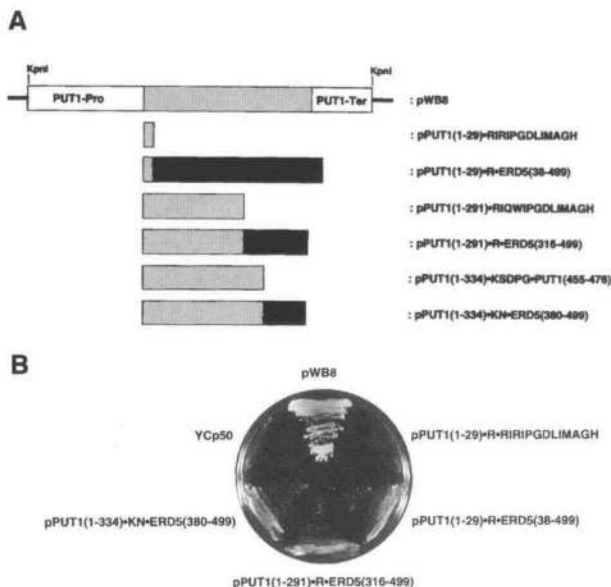

Figure 8. Complementation of a *put1* Mutant of Yeast by ERD5.

(A) Structures of the fusion and deletion genes. Three fusion genes of *PUT1* plus *ERD5* and three deleted *PUT1* genes were constructed by PCR. Shaded boxes indicate parts of coding regions derived from the structural gene for PUT1. Black boxes indicate parts of the coding region derived from ERD5 cDNA. PUT1-Pro indicates the 5′ noncoding region of the *PUT1* gene (1010 bp), and PUT1-Ter indicates the 3′ noncoding region of the *PUT1* gene (442 bp) (Wang and Brandriss, 1987). KpnI indicates the restriction site for KpnI introduced by PCR.
(B) Growth of *S. cerevisiae put1* mutant C15-1A on proline-containing medium without ammonium sulfate. Mutants transformed with the indicated plasmids were grown on agar-solidified (2%) medium that contained yeast nitrogen base without ammonium sulfate and amino acids (0.17%), L-tryptophan (0.004%), glucose (2%), and L-proline (0.5%) at 30°C for 3 days.

DISCUSSION

ERD5 as a cDNA for Proline Dehydrogenase

In this study, we demonstrate that ERD5, an Arabidopsis cDNA clone for a gene whose expression is upregulated after 1 hr of dehydration, encodes proline dehydrogenase, an enzyme involved in proline metabolism. This conclusion is based on the following evidence: The amino acid sequence identity of the ERD5 protein, as predicted from its nucleotide sequence, is similar to those of precursors to proline oxidase of yeast and Drosophila (Figure 2). Furthermore, the highly conserved regions at the C terminus found in these proteins (residues 307 to 354 and 426 to 485 of ERD5) are also found in the PutA protein of E. coli. PutA is a multifunctional protein that functions not only as a proline dehydrogenase but also as a P5C dehydrogenase and as a repressor of *put* genes (Ling et al., 1994). Therefore, these conserved regions seem to be functional domains of proline oxidase (dehydrogenase), for example, catalytic regions. Also, the amino acid sequence of the *ERD5*-encoded protein includes a putative signal for mitochondrial localization at the N terminus (Figure 1), and the

Table 1. Levels of Proline Oxidase in Plasmid-Bearing Transformants of the Mutant Strain C15-1A

Construct	Specific Activity of Proline Oxidase on the Indicated Nitrogen Source[a]	
	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$ + Proline
YCp50	0.27	0.21
pWB8	0.21	0.96
pPUT1(1-29)·RIRIPGDLIMAGH	0.33	0.30
pPUT1(1-29)·R·ERD5(38-499)	0.24	0.42
pPUT1(1-291)·RIQWIPGDLIMAGH	0.24	0.27
pPUT1(1-291)·R·ERD5(316-499)	0.27	0.84
pPUT1(1-334)·KSDPG·PUT1(455-476)	0.33	0.30
pPUT1(1-334)·KN·ERD5(380-499)	0.24	0.45

[a] Units of specific activity are given in nanomoles of P5C formed per minute per milligram of protein. Ammonium sulfate and proline were supplied at 0.2 and 0.1%, respectively.

ERD5 protein indeed was detected immunologically in a mitochondrial fraction from Arabidopsis cells (Figure 7C). This result reflects the previously reported localization of proline dehydrogenase on the matrix side of the inner membrane of mitochondria (Elthon and Stewart, 1981).

In addition, expression of the ERD5 gene was repressed by lengthy dehydration and was induced by rehydration after dehydration (Figure 3). This pattern of expression was the opposite of that of the gene for P5CS. Expression of this gene was induced by dehydration and repressed by rehydration (Figures 4A and 4B). Because the gene for P5CS encodes a key enzyme in the biosynthesis of proline from glutamate, this negative correlation between the patterns of expression of the two genes can be explained satisfactorily if the ERD5 gene encodes proline dehydrogenase, the first enzyme involved in the conversion of proline to glutamate. Moreover, proline dehydrogenase activity reportedly has been repressed by dehydration and activated by rehydration (Rayapati and Stewart, 1991), as has the expression of the ERD5 gene.

Both expression of its gene and activity of proline oxidase in yeast were reported to be induced by exogenous proline (Wang and Brandriss, 1987). In E. coli and Salmonella typhimurium, utilization of proline requires the two divergently transcribed genes that form the put operon: the putP gene, which encodes the major proline permease; and the putA gene, which encodes a multifunctional protein with proline oxidase activity (Maloy, 1987). The expression of this put operon also is induced by exogenous proline. The expression of the ERD5 gene was induced by exogenously supplied proline in Arabidopsis plants (Figure 5). The accumulation of the ERD5 protein in mitochondria of cultured cells also was induced by the addition of proline to the culture medium (Figure 7C). Therefore, the induction by exogenous proline is common to the ERD5 gene in Arabidopsis and to genes for proline oxidases in yeast, E. coli, and S. typhimurium.

Finally, a fusion gene for the PUT1 and ERD5 proteins complemented a put1 mutation in yeast, allowing mutant yeast cells to use proline as a nitrogen source and to grow on proline-containing medium without ammonium sulfate (Figure 8B and Table 1). It is unclear why the ERD5 gene alone, driven by a galactose-inducible promoter, failed to complement the put1 mutation, whereas the fusion gene driven by the PUT1 gene promoter complemented the mutation. One possible explanation is that the targeting signal for localization to the mitochondria of the ERD5 protein failed to function properly in yeast cells. Another explanation is that the galactose-inducible promoter in the multicopy-type plasmid pNV7 was not suitable for this complementation test, even though many complementation experiments have been performed successfully with this type of vector.

Expression of the ERD5 Gene

In dehydrated plants, the accumulation of proline occurs as the result of both the activation of proline biosynthesis and the inactivation of proline degradation, whereas a decrease in the accumulation of proline occurs as a result of both the inactivation of proline biosynthesis and the activation of proline degradation in rehydrated plants (Hellebust, 1976; Rayapati and Stewart, 1991; Delauney and Verma, 1993; Yoshiba et al., 1995). The decrease in proline dehydrogenase activity in water-stressed plants and the increase in this activity in rehydrated plants were observed by Sells and Koeppe (1981) and by Rayapati and Stewart (1991). They postulated that the decrease in activity might be caused by a specific change in the inner membrane of mitochondria that is unique to proline oxidation. The results in our study demonstrate that both the inactivation in dehydrated plants and the activation in rehydrated plants of proline dehydrogenase are regulated at the level of mRNA accumulation. Therefore, the activity of proline dehydrogenase is regulated not only by the level of the enzyme but also by the level of gene expression in plants.

The decrease of proline content in rehydrated plants occurred more rapidly than did the accumulation of proline in dehydrated plants, as shown in Figure 4. The increase in the accumulation of proline occurred after the accumulation of the P5CS mRNA had reached a maximum level in dehydrated plants, whereas the decrease in proline content occurred simultaneously with increases in the level of the ERD5 mRNA. The absence of a lag period between the decrease in the level of proline and the increase in the level of the ERD5 transcript suggests the existence of another pathway(s) for proline degradation in addition to that catalyzed by proline dehydrogenase or, alternatively, the rapid activation of proline dehydrogenase enzyme.

Expression of the ERD5 gene was induced by proline and repressed by osmotic stress. When rosette plants were incubated in medium that contained L-proline, expression of the

ERD5 gene was induced within 1 hr (Figure 5C). In contrast, expression of the *ERD5* gene was not induced in plants that had been dehydrated for 10 to 24 hr and that had accumulated high concentrations of L-proline. Expression of the *ERD5* gene might be induced by an elevated level of intracellular L-proline and repressed by osmotic stress. This hypothesis can explain the results shown in Figure 4. Dehydration of plants caused osmotic stress and subsequently led to elevated levels of proline in the plant cells. The proline that had formed several hours after the onset of stress by biosynthesis de novo could not induce expression of the *ERD5* gene because of repression by osmotic stress. When plants were rehydrated, expression of the *ERD5* gene became inducible by L-proline because there was no osmotic stress or repression.

We do not know why D-proline induced more stable expression of the *ERD5* gene than did L-proline, as shown in Figures 5C and 5E. It is possible that because L-proline is metabolizable whereas D-proline is not, D-proline is more stable in plant cells and subsequently acts more stably to induce expression of the *ERD5* gene. Another explanation might involve the allosteric effects of D-proline on proline perception molecules in cells, such as sensor proteins and transcription factors, whose activities are modulated by the binding of proline.

Proline appears to function as a signal for induction of the *ERD5* gene, but there seems to be another signal(s) that induces expression of the *ERD5* gene. A transient increase in the level of the *ERD5* transcript was observed after dehydration or heat or cold stress for 1 to 2 hr (Figure 3). Expression of the *ERD5* gene was also induced when plants were transferred to a medium with low osmotic pressure or to a medium without sucrose (Figure 5A). Because no transient increase in the level of intercellular proline was detected during these treatments (data not shown), such induction of the *ERD5* gene seemed to be caused not by a proline signal but by another factor(s) that resulted from these various stresses.

In this study, we characterized the ERD5 cDNA as a cDNA for a precursor to proline dehydrogenase of Arabidopsis. The conversion of proline to glutamic acid is the reverse of the biosynthesis of proline from glutamate. Proline is synthesized from glutamate by the actions of two enzymes, P5CS and P5CR. Proline is acted on by proline dehydrogenase and then by P5C dehydrogenase to yield glutamate. The cDNAs for three of these enzymes in plants, namely, P5CS, P5CR, and proline dehydrogenase, have been isolated. Therefore, molecular characterization of P5C dehydrogenase is important if we are to understand the molecular mechanisms that regulate the proline content of plants. Because expression of the *ERD5* gene was inducible in rehydrated plants in which rapid degradation of proline occurred and because expression of the *ERD5* gene was detected in many cells of all organs examined, proline dehydrogenase seems to play a crucial role in degradation of proline in plants after termination of water stress. Analysis of the *cis*- and *trans*-acting factors that control expression of the gene encoding proline dehydrogenase should help us to understand the molecular mechanisms of the regulation of gene expression by proline and osmotic stress.

METHODS

Plant Materials

Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on vermiculite beds or axenically on GM agar medium (Valvekens et al., 1988) for ~4 weeks, as described by Kiyosue et al. (1993b, 1994a). The T87 cell line, derived from Arabidopsis, which grows in suspension culture, was kindly provided by M. Axelos (CNRS-INRA, Castanet-Tolosan, France) via M. Pagès (CSIC, Barcelona, Spain) and subcultured at 2-week intervals in JPL medium (Axelos et al., 1992).

Analysis of DNA and Amino Acid Sequences

DNA sequences were determined by the dye primer cycle sequencing method with a DNA sequencer (model 373A; Applied Biosystems, Foster City, CA). The GENETYX (Software Development, Tokyo, Japan) and GeneWorks (IntelliGenetics, Inc., Mountain View, CA) software systems were used for the analysis of nucleotide and amino acid sequences.

Isolation of RNA and DNA from Arabidopsis

RNA and genomic DNA were isolated from whole rosette plants and cultured cells, as described by Kiyosue et al. (1993a).

Hybridization of RNA and DNA

Fragments of the ERD5 cDNA were labeled by the random primer method with ^{32}P-dCTP (110 TBq/mmol; Amersham, Aylesbury, UK) by using the random primed DNA labeling kit from Boehringer Mannheim. Fragments were hybridized with RNA and DNA, as described by Kiyosue et al. (1994a).

Dehydration, Rehydration, and Heat Shock and Cold Shock Treatments

Dehydration, heat shock, and cold shock treatments were performed as described by Kiyosue et al. (1993a, 1994a). For rehydration, 4-week-old rosette plants of Arabidopsis that had been dehydrated for 10 hr were transferred to water and incubated for 1 to 48 hr under dim light.

In Situ Hybridization

In situ hybridization analysis was performed with the digoxigenin-labeled RNA probe for ERD5, according to the protocol of de Almeida Engler et al. (1994) but with one modification, that is, the temperature for hybridization was decreased to 42 from 60°C. Photographs were taken under a light microscope (model BH-2; Olympus, Tokyo, Japan).

Production of Glutathione S-Transferase Fusion Proteins

Two primer sets, 5'-GATGGATCCGATCTCTCCGAT-3' plus 5'-TACGAA-TTCCCGGGGGATTAATCTCCTCTT-3' and 5'-CCGGGATCCTTAACC-

GCGGAA-3′ plus 5′-TACGAATTCCCGGGGATTAATCTCCTCTT-3′, were used for polymerase chain reactions (PCRs). Each pair of primers was used at 100 pmol. Ten nanograms of ERD5 cDNA as template and 2 units of Pfu DNA polymerase (Stratagene) were also used for each reaction. Forty cycles of denaturation, annealing, and polymerization were performed at 94°C for 1 min, 37°C for 2 min, and 72°C for 3 min, respectively. The amplified DNA fragments of ~1.1 kb and 500 bp were cloned into the pCR-Script vector (Stratagene), amplified in *Escherichia coli* cells, isolated, sequenced, and then cut out by BamHI and EcoRI restriction enzymes. These fragments were ligated to the pGEX2T vector (Smith and Johnson, 1988), and the recombinant plasmids, pGST·ERD5(64-499), the former, and pGST·ERD5(250-499), the latter, were used for the transformation of *E. coli* XL1-Blue cells. Production of recombinant proteins by these transformant cells was induced as described by Smith and Johnson (1988).

Gel Electrophoresis, Electroblotting, and Immunological Detection

Proteins were separated by SDS-PAGE (5% acrylamide stacking gel and 10% acrylamide separating gel; Laemmli, 1970) and then stained with Coomassie Brilliant Blue R 250 or transferred electrophoretically to a nitrocellulose filter in a solution of 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol at 10 mA for 30 min. For immunodetection, the nitrocellulose filter was agitated in 1% nonfat dry milk in PBS for 1 hr at room temperature and then in 0.2% (v/v) rabbit antiserum that had been raised against a peptide that corresponded to amino acids 480 to 494 of the predicted ERD5 protein (AHDRQLMRMELKRRL) in PBS for 1 hr. The blot was washed with 0.1% Tween 20 in PBS and then agitated in a 0.04% solution (v/v) of horseradish peroxidase–conjugated antibodies raised against rabbit IgG raised in goat (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS for 1 hr at room temperature. The immunoreactive protein on the blot was visualized by placing the blot in PBS that contained 0.03% 3,3-diaminobenzidine and 0.003% H_2O_2.

Preparation of Mitochondrial and Chloroplast Fractions from Cultured Cells of Arabidopsis

All analyses were performed at 4°C. Cultured cells of Arabidopsis (20 g fresh weight) that had been incubated in JPL medium (Axelos et al., 1992) plus 0.1 M D-proline for 0, 1, 2, 3, 7, or 10 days were collected and homogenized with a glass Teflon homogenizer in 40 mL of 50 mM Tris-HCl buffer, pH 8.0, that contained 0.33 M sucrose, 0.2% BSA, 0.1% ascorbic acid, 0.05% 2-mercaptoethanol, 5 mM EDTA, and 5 mM $MgCl_2$. The homogenate was filtered through two sheets of Miracloth (Calbiochem–Novabiochem, La Jolla, CA) and centrifuged first at 4000g for 15 min and then at 12,000g for 20 min. The final pellet was suspended gently in 10 mL of 10 mM KH_2PO_4 buffer, pH 7.2, that contained 0.25 M sucrose and 0.1% BSA, and the suspension was layered on a discontinuous Percoll density gradient. The discontinuous gradient was composed of 5 mL of 45% Percoll, 15 mL of 28% Percoll, and 5 mL of 5% Percoll in 10 mM KH_2PO_4 buffer, pH 7.2, plus 0.25 M sucrose and 0.1% BSA. Centrifugation was performed at 30,000g for 30 min (Nishimura et al., 1982). One-milliliter fractions were collected sequentially from the bottom of the tube, and an aliquot of each was used for an assay of cytochrome c oxidase (EC 1.9.3.1) and quantitation of chlorophyll. Cytochrome c oxidase was assayed as described by Nawa and Asahi (1971). Chlorophyll was extracted in 80% acetone

and quantitated by the method of Cosio et al. (1983). Protein concentrations were determined with a protein assay kit (Bio-Rad). The fractions at the interface between 45 and 28% Percoll (mitochondrial fraction) and between 28 and 5% Percoll (chloroplast fraction) were collected and examined immunologically for the presence of the ERD5 protein.

Construction of Fusion Genes from *PUT1* and *ERD5* Genes and of Partially Deleted Versions of the *PUT1* Gene

Four primer sets were used: 5′-CGCAGGTGGGTACCCATCTACTC-3′ and 5′-AGTGGATCCGAATTCTTGACACATAGGACCTCTT-3′; 5′-CGCAGG-TGGGTACCCATCTACTC-3′ and 5′-TAGGATCCACTGAATTCTACCGAC-ACATGATAT-3′; 5′-CGCAGGTGGGTACCCATCTACTC-3′ and 5′-TAGGAT-CCACTGAATTCTACCGACACATGATAT-3′; and 5′-TTGCAAGAAAAC-GGGGATCCCGGGAGATCT-3′ and 5′-A(GATC)TCTAGAGGTACCCCAA-AATCCTTACAAAG-3′. The primer sets were used for PCRs to amplify four fragments, P1, P2, P3, and T1. Each pair of primers was used at 100 pmol. Ten nanograms of pWB8 DNA, as template, and 2 units of Pfu DNA polymerase also were used for each reaction. Forty cycles of denaturation, annealing, and polymerization were performed at 94°C for 1 min, 37°C for 2 min, and 72°C for 3 min, respectively. The amplified DNA fragments (P1, P2, P3, and T1) were cloned into the pCR-Script vector, amplified in *E. coli* cells, isolated, and sequenced. Inserts then were cut out by both BamHI and KpnI for P1, P2, and P3 or both BamHI and XbaI for T1. The T1 fragment, which corresponds to the PUT1-Ter region in Figure 7A, was ligated to the P1, P2, or P3 fragment and then cloned in the pBluescript SK+ vector (Stratagene) that had been treated with both KpnI and XbaI to generate plasmids pP1T1, pP2T1, and pP3T1, respectively. The plasmids were amplified in *E. coli* cells, isolated, and sequenced. The inserts were cut out by KpnI, and the resultant fragments were cloned into the KpnI site of the YCp50 vector (Parent et al., 1985) to yield pPUT1(1-29)·RIRIPG-DLIMAGH, pPUT1(1-291)·RIQWIPGDLIMAGH, and pPUT1(1-334)·KSDPG·PUT1(455-476), respectively.

Three fragments corresponding to the coding region of *ERD5* (E1, E2, and E3) also were amplified by PCR under the same conditions as given above with the following exceptions. The primer sets used were 5′-CGTCCCGGGAATTCTCTCCT-3′ and 5′-ATTTAGCCCCGG-GAAATGTTCTTACC-3′; 5′-GTTTACAACAGAATTCAGGC-3′ and 5′-ATT-TAGCCCCGGGAAATGTTCTTACC-3′, and 5′-GTAGCTTGGCGAATT-CCCTG-3′ and 5′-ATTTAGCCCCGGGAAATGTTCTTACC-3′, respectively. Ten nanograms of ERD5 cDNA was used as template. The amplified DNA fragments (E1, E2, and E3) were cloned into the pCR-Script vector, amplified in *E. coli* cells, isolated, and sequenced. The inserts were then cut out by both EcoRI and SmaI and ligated to pP1T1, pP2T1, or pP3T1 that had been treated with both EcoRI and SmaI to yield pP1E1T1, pP2E2T1, and pP3E3T1, respectively. Each plasmid was digested with KpnI, and the KpnI fragment corresponding to a fusion of the *PUT1* and *ERD5* genes was cloned into the KpnI site of the YCp50 vector to generate pPUT1(1-29)·R·ERD5(38-499), pPUT1(1-291)·R·ERD5(316-499), and pPUT1(1-334)·KN·ERD5(380-499), respectively.

Complementation of a *put1* Mutant Strain of *Saccharomyces cerevisiae*

Ura[+] transformants were selected on synthetic medium that contained 2% glucose but no uracil at 30°C. The transformed yeast cells were subsequently grown at 30°C on agar (2%) medium that contained yeast

nitrogen base without ammonium sulfate and amino acids (YNB-AA/AS; Difco Laboratories, Detroit, MI; 0.17%), L-tryptophan (0.004%), glucose (2%), and L-proline (0.5%). Photographs were taken under a light microscope (model BH-2; Olympus) after incubation for 3 days on the above-mentioned medium.

Assay for Proline Oxidase Activity

Transformed yeast cells were grown in liquid medium that contained YNB-AA/AS (0.17%), L-tryptophan (0.004%), glucose (2%), and ammonium sulfate (0.1%), with or without L-proline (0.1%). Cells at the logarithmic phase of growth were harvested and permeabilized by treatment with liquid nitrogen. The enzyme was assayed as described by Brandriss and Magasanik (1979).

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