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Cloning and characterization of a cDNA encoding the chloroplastic copper/zinc-superoxide dismutase from pea

(cDNA library/nucleotide sequence/chloroplast transport/transit sequence/photooxidative stress)

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ABSTRACT A cDNA clone encoding the chloroplastic copper/zinc-superoxide dismutase of pea (*Pisum sativum* L.) was isolated from a cDNA library constructed in λgt11 from leaf mRNA. Nucleotide sequence analysis of the 875-base-pair clone revealed that it contained the complete coding sequence of the mature superoxide dismutase isozyme subunit, along with sequence information for a 48-amino acid N-terminal transit peptide. The deduced amino acid sequence of the mature subunit proved to be 64–87% homologous with amino acid sequences of copper/zinc-superoxide dismutases from other plant species. *In vitro* transcription, followed by cell-free translation, of the cDNA resulted in the formation of a 23.5-kDa precursor polypeptide, which, upon incubation with isolated pea chloroplasts, was imported and processed to its mature subunit molecular mass of 17.4 kDa.

Superoxide dismutases (SODs; EC 1.15.1.1) are a ubiquitous and diverse class of metalloenzymes found in all aerobic and some anaerobic organisms. Catalyzing the dismutation of superoxide radical anions to dioxygen and hydrogen peroxide, SODs are important in the protection of cells and organelles from oxidative damage. In chloroplasts, the superoxide radical is produced by the univalent reduction of dioxygen during photosynthetic electron transport, particularly under conditions where CO_2 is limited and light intensity is high (1). Because superoxide has been postulated as a reactant in the formation of the highly reactive hydroxyl radical (1), SODs may play a central role in protecting chloroplast proteins and membranes against damage from reactive oxygen species.

SODs are categorized into two families: one contains copper and zinc in the active site (Cu/Zn-SOD), whereas the active site of the other contains either manganese (Mn-SOD) or iron (Fe-SOD) (2). The copper/zinc enzyme is the most prevalent SOD in plant tissue. In leaves, the bulk of Cu/Zn-SOD activity is contained in chloroplasts, with the remainder in the cytoplasm and, in some species, in the mitochondrial intermembrane space (3, 4). Plant tissue also contains Mn-SOD, located in the mitochondrial matrix. In maize (5) and yeast (6), Mn-SOD subunits are synthesized in the cytoplasm as larger molecular mass precursors, which are posttranslationally processed upon import into mitochondria. Nucleotide sequence analysis of a gene encoding the yeast Mn-SOD showed that it contains coding information for a 27-amino acid transit peptide (7). An Fe-SOD has also been reported in some plant species (4).

Cu/Zn-SODs usually exist as multiple isozymes in photosynthetic cells of higher plants, with at least one isozyme located in the chloroplast. Isozymes of Cu/Zn-SOD have been purified from a variety of plant sources. Amino acid sequences of the spinach chloroplastic (8) and cabbage cytosolic (9) isozymes have been published, as well as the cDNA nucleotide and deduced amino acid sequences of the maize cytosolic SOD 2 (10). Pea (*Pisum sativum* L.) leaves contain two Cu/Zn-SOD isozymes, one chloroplastic and the other cytosolic, which differ in subunit molecular mass, isoelectric point, and amino acid composition (11). We report the isolation of a cDNA clone that encodes the chloroplastic isozyme of Cu/Zn-SOD from pea.† Its identity has been confirmed by nucleotide sequence analysis, along with *in vitro* transcription, followed by cell-free translation and transport into isolated pea chloroplasts.

MATERIALS AND METHODS

Preparation of Antisera. The chloroplastic and cytosolic pea Cu/Zn-SODs were identified by the gel activity assay of Beauchamp and Fridovich (12) following electrophoresis of soluble leaf proteins and chloroplast stromal proteins on a nondenaturing polyacrylamide [12% (wt/vol)] gel. The gel system was as described by Laemmli (13) but lacked $NaDodSO_4$. To determine subunit molecular masses of the two isozymes, bands containing activity were excised from the native gel and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis (13). The Cu/Zn-SOD isozymes were then partially purified from 4-week-old pea leaves and stems (Burpee Progress no. 9) by ammonium sulfate fractionation and ion-exchange chromatography on DEAE-cellulose (DE23 and DE52, Pharmacia) as described by Duke and Salin (11). The proteins were further purified by preparative $NaDodSO_4$/polyacrylamide gel electrophoresis in 12% (wt/vol) acrylamide gels, and the two bands ascertained as the cytosolic and chloroplastic isozyme subunits were excised and used as antigens for generation of polyclonal antisera in rabbits.

Antibodies were affinity purified by a modification of a published method (14). The antiserum raised against the pea cytosolic Cu/Zn-SOD was incubated with a semipurified preparation of pea Cu/Zn-SODs immobilized onto nitrocellulose. Unbound antibodies were removed by extensive washing of the filter with 10 mM Tris·HCl, pH 7.5/150 mM NaCl; bound antibodies were then eluted with a solution of 5 mM glycine hydrochloride, pH 2.3/0.5 M NaCl/0.1 mg of bovine serum albumin per ml and then immediately neutralized with a 1/10th volume of 0.5 M Na_2HPO_4. Electrophoretic transfer of proteins to nitrocellulose and immunoblot analysis were performed using published procedures (15).

Screening of the λgt11 Library. A cDNA library, constructed from pea leaf mRNA (16) in the expression vector λgt11, was generously provided by J. Stephen Gantt (University of Minnesota, Saint Paul). The library was screened according to the method of Huynh, Young and Davis (17),

Abbreviations: SOD, superoxide dismutase; Cu/Zn-SOD, copper/zinc-SOD; Mn-SOD, manganese-SOD; Fe-SOD, iron-SOD.

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†The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J04087).

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using the *Escherichia coli* host strain Y1090, at a density of 30,000 plaques per 82-mm (diameter) plate. Plaques containing proteins cross-reacting with affinity-purified antibodies against pea Cu/Zn-SOD were detected enzymatically with goat anti-rabbit IgG conjugated to alkaline phosphatase. Recombinant phage DNA was purified by a method previously described (18).

Isolation and Cloning of cDNA. Recombinant λgt11 DNA was digested with *Eco*RI to release the cDNA inserts, and the fragments were separated by electrophoresis in 1% agarose gels. Intact cDNA inserts, or restriction fragments of selected inserts, were collected on DEAE-cellulose membrane (NA 45, Schleicher & Schuell), eluted, and ligated into pUC12 or pUC13, which had been linearized with appropriate restriction endonucleases and treated with alkaline phosphatase. Transformations of *E. coli* strains JM109, DH5α, and HB101 were carried out by using the rapid transformation procedure of Hanahan (19).

DNA Sequence Analysis. Restriction fragments cloned into pUC12 and pUC13 were sequenced by the dideoxy chain-termination method (20), using modifications for double-stranded plasmid templates developed by Hattori and Sakaki (21). Plasmid DNA was isolated by the alkaline lysis method (22). Further purification in some cases, by Sephacryl S-1000 (Pharmacia) column chromatography, was according to the method of Bywater *et al.* (23). Sequencing reactions were carried out either with the large (Klenow) fragment of DNA polymerase I or "Sequenase" (United States Biochemical, Cleveland) with dATP[γ-^{35}S] (Amersham; 650 Ci/mmol; 1 Ci = 37 GBq) as the radiolabeled nucleotide.

***In Vitro* Transcription/Translation of cDNA.** The cDNA inserts were cloned into the *Eco*RI site of the transcription vector pSP64, and plasmid DNA was purified by CsCl gradient centrifugation. The plasmid was linearized at a unique *Pvu* II site 180 base pairs (bp) downstream from the *Eco*RI site. Transcriptions were performed with SP6 RNA polymerase (Bethesda Research Laboratories) using the protocol of the "Riboprobe" *in vitro* transcription system (Promega Biotec, Madison, WI), according to instructions for transcription of translatable mRNA. Transcripts were extracted with equal volumes of phenol and chloroform and were then precipitated with 2.5 volumes of absolute ethanol and sodium acetate at a final concentration of 0.3 M. The transcript (0.1–1.0 μg) was translated in a wheat germ cell-free protein synthesizing system. The wheat germ (General Mills) S30 fraction was prepared by the method of Roberts and Paterson (24). The translation was carried out as described by Gantt and Key (25) using 5 μCi of uniformly labeled [^{3}H]leucine (ICN, 110 Ci/mmol) per 25-μl translation reaction mixture.

RNA Blot Hybridizations. Pea leaf RNA was isolated according to methods described (26). Pea leaf total RNA and mRNA obtained from *in vitro* transcription of cloned cDNA were denatured and fractionated by electrophoresis in 1.5% agarose formaldehyde gels and then transferred to nitrocellulose filters (27). Filters were probed with denatured DNA restriction fragments that had been labeled by nick-translation (27) with [α-^{32}P]dATP (ICN, 3500 Ci/mmol). Hybridizations were carried out at 42°C for 12–15 hr in 50% (vol/vol) deionized formamide, 5× SSPE (50 mM sodium phosphate, pH 7.4/900 mM NaCl/5 mM EDTA), 0.1% $NaDodSO_4$, 0.2 mg of Ficoll per ml, 0.2 mg of bovine serum albumin per ml, and 100 μg of denatured calf thymus DNA per ml. Washes were 4 × 15 min in 0.1× SSPE/0.1% $NaDodSO_4$ at 50°C. Filters were air dried and autoradiographed at −70°C with intensifying screens.

Import of *in Vitro* Translation Products into Isolated Pea Chloroplasts. Intact chloroplasts were prepared from 9- to 12-day-old pea seedlings (Burpee Progress no. 9) as described (28). The procedure of Pain and Blobel (29) was followed for the import assays. Chloroplasts, diluted in import buffer, were preincubated 15 min in darkness. Assays were then conducted in the light from 5 to 30 min at 25°C, with rotary shaking (110 rpm), without the addition of exogenous ATP. The import reaction mixture was as described in ref. 29 but contained 10 mM leucine rather than methionine, chloroplasts equivalent to 100 μg of chlorophyll, and 8 μl of *in vitro* translation products (20,000 cpm/μl). Dark control assays were kept covered with aluminum foil for the 30-min assay period. Nigericin-treated assays were incubated for 30 min in the presence of 50 nM nigericin. For protease and detergent treatments, chloroplasts were pelleted after the 30-min assay and then gently resuspended in 300 μl of cold 50 mM Hepes/KOH, pH 7.7/0.33 M sorbitol containing 50 μg of thermolysin per ml, either alone or with 0.3% (vol/vol) Triton X-100; this was followed by a 30-min incubation on ice. Chloroplasts were recovered by centrifugation and then lysed by resuspending in 2 mM EDTA. Membranes were pelleted, and proteins from the supernatants were precipitated with 1/10th volume of 100% trichloroacetic acid, washed with acetone, and neutralized with 0.1 M Na_2CO_3. Samples were denatured and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography.

Computer Analyses. Computer analyses of DNA and protein sequences were performed with programs from the University of Wisconsin Genetics Computer Group Sequence Analysis software package (30).

RESULTS AND DISCUSSION

Preparation of Antisera. The nondenaturing polyacrylamide gel activity assay (12) of soluble proteins from pea leaves and soluble proteins from isolated pea chloroplasts showed that the more anodic SOD isozyme was associated exclusively with chloroplasts (Fig. 1*B*). When excised and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, the chloroplastic isozyme was found to have an estimated subunit

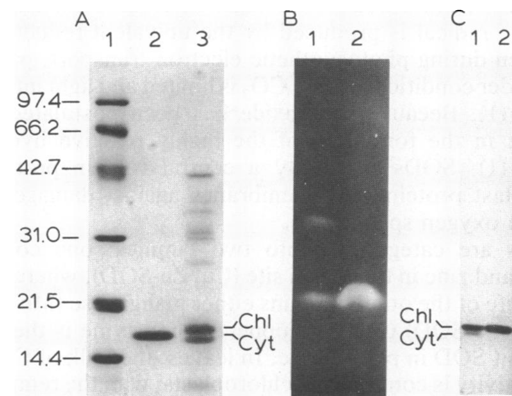

FIG. 1. Preparation and analysis of pea Cu/Zn-SODs for use as antigens. (*A*) Coomassie blue-stained $NaDodSO_4$/polyacrylamide gel electrophoretogram of semipurified Cu/Zn-SODs. Lane 1, molecular mass standards (shown in kDa); lane 2, bovine erythrocyte Cu/Zn-SOD (Boehringer Mannheim); lane 3, semipurified pea leaf Cu/Zn-SOD isozymes; chloroplastic (Chl) and cytosolic (Cyt) isozymes are indicated. (*B*) Nondenaturing polyacrylamide gel activity assay of total soluble pea leaf proteins (lane 1) and soluble chloroplast stromal proteins (lane 2). Pea leaves were homogenized in 50 mM Tris·HCl (pH 7.8) and centrifuged 10 min at 10,000 × *g*; the supernatant was used for gel assay. Chloroplast stromal proteins were isolated from intact chloroplasts, which were lysed and centrifuged 15,000 × *g* for 10 min; the supernatant was assayed. (*C*) Immunoblot showing cross-reactivity of affinity-purified antibodies, raised against the pea cytosolic Cu/Zn-SOD, with both isozymes of Cu/Zn-SOD from pea. Samples were prepared as in *B*, except that proteins were precipitated from supernatants by 10% (wt/vol) trichloroacetic acid and then resuspended and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis in a 10–20% (wt/vol) acrylamide gradient. Lane 1, extract of total soluble pea leaf protein; lane 2, extract of chloroplast stromal proteins.

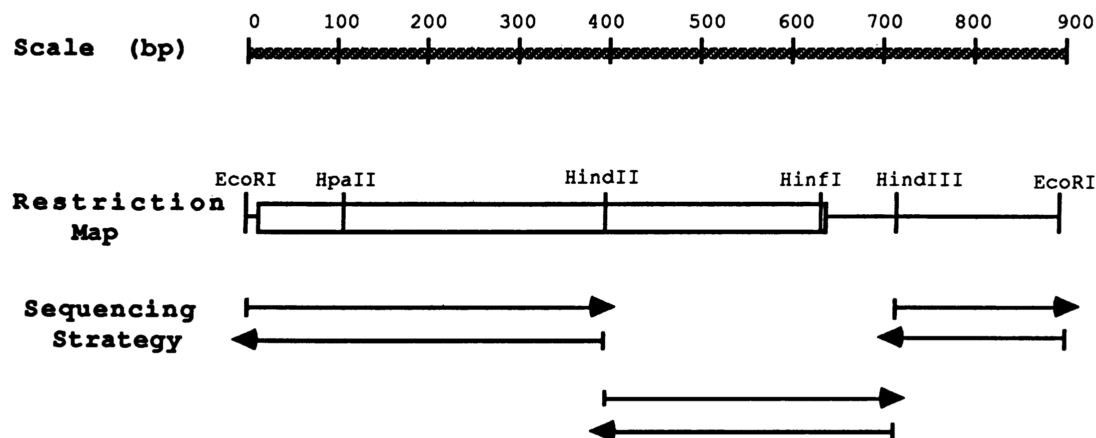

FIG. 2. Restriction map of the cDNA encoding the pea chloroplastic Cu/Zn-SOD. Restriction sites are indicated. The bar denotes the coding region; the single line indicates the noncoding region; arrows show the direction and distance of sequencing.

molecular mass of 17.4 kDa, whereas the molecular mass of the cytosolic isozyme subunit was estimated as 16.8 kDa. These findings, in close agreement with previous reports (11), were used to identify the chloroplastic and cytosolic Cu/Zn-SOD polypeptides in preparative-scale purification of the proteins by NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 1*A*). The polypeptides were used as antigens for polyclonal antisera production in rabbits. As shown in Fig. 1*C*, antibodies raised in rabbits against the pea cytosolic Cu/Zn-SOD cross-reacted with the cytosolic and chloroplastic isozymes, as determined by immunoblot analysis.

Screening of the λgt11 Library. Immunological screening of a λgt11 library constructed from pea leaf mRNA showed that 18 of 350,000 plaques contained protein that cross-reacted with the affinity-purified Cu/Zn-SOD antibodies (data not shown). Two strongly cross-reacting plaques were rescreened and isolated to homogeneity. The cDNA inserts were removed from the vector by *Eco*RI digestion and cloned into pUC13; the recombinant plasmids were designated pA2 and pC3. Both inserts were ≈900 bp, and their restriction endonuclease cleavage patterns were identical (Fig. 2).

DNA Sequence Analysis. *Hin*dII and *Hin*dIII restriction cleavage sites were used for subcloning of pA2 fragments into pUC12 and pUC13 for nucleotide sequence analysis (Fig. 2). The nucleotide sequence and deduced amino acid sequence of the cDNA are shown in Fig. 3. The cDNA contains an open reading frame of 609 nucleotides, flanked 5′ by 10 bases of noncoding sequence and 3′ by 206 bases of noncoding sequence and a 50-base poly(A) tail. A putative polyadenylylation signal (AATAAA) appears 18 bases upstream from the start of the poly(A) tail. The open reading frame contains coding information for the mature subunit of a Cu/Zn-SOD, the sequence of which is highly conserved among plant species (see Fig. 4). In addition, the cDNA encodes an N-terminal extension of 48 residues, which constitutes the transit peptide. The transit sequence is rich in basic, hy-

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                                                                                          Nucleotide
                         Met Ala Ser Gln Thr Leu Val Ser Pro Ser Pro Leu Ser Ser His Ser Leu Leu Arg
      GAATTCGGGCAACTAGCA ATG GCT TCA CAA ACT CTC GTC TCA CCT TCA CCT CTC TCT TCT CAC TCT CTT CTC CGA     67

Thr Ser Phe Ser Gly Val Ser Val Lys Leu Ala Pro Gln Phe Ser Thr Leu Ala Thr Ser Asn Phe Lys Pro Leu
ACA TCT TTC TCC GGC GTC TCC GTC AAG CTC GCT CCC CAA TTC TCA ACC CTT GCA ACT TCC AAT TTC AAA CCT CTC    142
                 ↓
Thr Val Val Ala Ala Ala Lys Lys Ala Val Ala Val Leu Lys Gly Thr Ser Ala Val Glu Gly Val Val Thr Leu
ACC GTA GTT GCG GCT GCC AAG AAA GCC GTC TCT GTC CTT AAG GGC ACA TCC GCC GTC GAA GGT GTC GTC ACT CTC    217

Thr Gln Asp Asp Glu Gly Pro Thr Thr Val Asn Val Arg Ile Thr Gly Leu Thr Pro Gly Leu His Gly Phe His
ACT CAA GAC GAT GAA GGT CCA ACA ACA GTT AAT GTT CGT ATC ACT GGC CTT ACT CCA GGG CTT CAT GGT TTT CAC    292

Leu His Glu Tyr Gly Asp Thr Thr Asn Gly Cys Ile Ser Thr Gly Pro His Phe Asn Pro Asn Lys Leu Thr His
CTA CAT GAG TAT GGT GAT ACC ACA AAT GGG TGT ATC TCA ACA GGA CCA CAT TTT AAT CCC AAC AAG TTG ACA CAT    367

Gly Ala Pro Glu Asp Glu Ile Arg His Ala Gly Asp Leu Gly Asn Ile Val Ala Asn Ala Glu Gly Val Ala Glu
GGT GCT CCT GAA GAT GAA ATC CGT CAT GCG GGT GAC CTG GGA AAC ATA GTT GCT AAT GCT GAA GGA GTT GCA GAG    442

Ala Thr Ile Val Asp Asn Glu Ile Pro Leu Thr Gly Pro Asn Ser Val Val Gly Arg Ala Leu Val Val His Glu
GCG ACA ATC GTG GAC AAT CAG ATA CCA CTC ACT GGC CCC AAT TCA GTC GTT GGA AGA GCC TTA GTG GTT CAC GAG    517

Leu Gln Asp Asp Leu Gly Lys Gly Gly His Glu Leu Ser Leu Ser Thr Gly Asn Ala Gly Gly Arg Leu Ala Cys
CTT CAA GAT GAC CTT GGA AAG GGT GGA CAT GAA CTT AGT TTG AGC ACT GGA AAT GCT GGT GGA AGA TTA GCT TGT    592

Gly Val Val Gly Leu Thr Pro Val  *
GGT GTG GTT GGC TTG ACT CCA GTA TAA ATGCTTCAATGCTTTTGCACCAGCCTTGTTATTTTAACTGATGTTTGATTTCTTCATGT    678

TATCCTTGTTTTATGAAGCTTTACTGTTATTTGTTTTCTTCAATTAGCAATGTAAAGATTTTAATGTGTGAAAACATGACAGTCCTCTACTAG     771

ATAGTTTCATCATGCAGTCAGGTGTGTTTCTTCCCTAATAAAGTTTAATTTCTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA    865

AAAAAAAAAACCGAATTC
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FIG. 3. Nucleotide and deduced amino acid sequences of the cDNA encoding the pea chloroplastic Cu/Zn-SOD. Consensus sequences of the translation start site and polyadenylylation signal are underlined. The arrow indicates the putative transit peptide cleavage site. The asterisk indicates the stop codon.

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                                            20                                      40
Pea (chl)        A A K K A V - - - A V L K G T S A V E G T V T L I Q E D E G P T T V S V R I T G
Spinach (chl)    A T K K A V - - - A V L K G T S N V E G V V T L T Q E D D G P T T V N V R I S G
Maize (cyt)    M V - - K A V - - - A V L A G T D - V K G T I F F S Q E G D G P T T V T G S I S G
Cabbage (cyt)    A - - K A V N S S A V L I G - - - V K G T I F F T H E G N G A T T V T G T V S G

                             *   *                 60             *              *          80
Pea              L T P G T H G F H L H E Y G D T T N G C I S T G P H F N P N K A L T H G A P E D
Spinach          L A P G K H G F H L H E F G D T T N G C M S T G P H F N P D K - K T H G A P E D
Maize            L K P G L H G F H V H A L G D T T N G C M S T G P H F N P V G - K E H G A P E D
Cabbage          L R P G L H G F H V H A L G D N T N G C M S T G P H F N P D G - K T H G A P E D

                     *   *                        100                                      120
Pea              E N R H A G D L G N I V A N A E G V A E A T I V D N Q I P L T G P N S V V G R A
Spinach          E V R H A G D L G N I V A N T D G V A E A T I V D N Q I P L T G P N S V V G R A
Maize            E D R H A G D L G N V T A G E D G V V N V N I T D S Q I P L A G P H S I I G R A
Cabbage          A N R H A G D L G N I I V G D D G T A T F T I T D S Q I P L S G P N S I V G R A

                       *                          140
Pea              L V V H E L Q D D L G L G G H E L S L S T G N A G G R L A C G V V G L T P V
Spinach          L V V H E L E D D L G L G G H E L S P T T G N A G G R L A C G V V G L T P V
Maize            V V V H A D A D D L G K G G H E L S K S I G N A G G R V A C G I I G L Q G
Cabbage          I V V H A D P D D L G K G G H E L S L S T G N A G G R V A C G I I G L Q G
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FIG. 4. Comparison of the deduced amino acid sequence of the mature pea chloroplast Cu/Zn-SOD with sequences from other plant species. The deduced amino acid sequence of the pea isozyme is compared with the sequence of the chloroplastic Cu/Zn-SOD from spinach (8), the maize cytosolic SOD 2 (deduced from cDNA nucleotide sequence) (10), and a cytosolic Cu/Zn-SOD from cabbage (9). Regions of complete homology are boxed. Residues coordinating copper and zinc are indicated with asterisks.

droxylated, and hydrophobic amino acids and lacks negatively charged residues, all characteristic of known transit peptides (31). The DNA sequence at the translation start site (AGCAATGG) closely matches the consensus sequence (AACAATGG) reported for this region in plants (32), specifically dicotyledonous species (33).

Amino Acid Sequence Comparison. Displayed in Fig. 4 is a comparison of amino acid sequences of Cu/Zn-SODs from four plant species, either determined by Edman degradation (8, 9) or deduced from nucleotide sequences of cDNAs (ref. 10; this report). The overall sequence homology among the four species is 54%. Aside from the transit sequence of the pea SOD, the chloroplastic and cytosolic Cu/Zn-SOD sequences are quite similar with respect to length and alignment of the homologous regions. This similarity extends to cytosolic Cu/Zn-SODs from other eukaryotes (2). In contrast, the Cu/Zn-SOD from *Photobacterium leognathi* (34) contains extended regions of nonhomologous amino acid sequence, and the human extracellular Cu/Zn-SOD (35) possesses unique N- and C-terminal extensions, as well as a signal sequence. It should be noted that the seven ligands coordinating the copper and zinc, as well as the two cysteines that form the single disulfide bridge, are conserved, as they are among all known Cu/Zn-SODs. The pea and spinach sequences of the mature chloroplastic isozymes are 87% identical, whereas the maize (deduced) and cabbage amino acid sequences (both cytosolic isozymes) are 74% identical. However, when the pea sequence is compared with either the maize or cabbage sequence, the homology decreases to 65% and 64%, respectively. In a comparison of Cu/Zn-SOD amino acid compositions from several plant species, Kwiatowski and Kaniuga (36) noted that chloroplast isozymes are more similar to each other than are cytosolic isozymes and that the similarity decreases further when chloroplastic and cytosolic isozymes are compared. The amino acid sequence comparisons made here support that observation, although the deduced amino acid composition of the pA2 clone differs (with respect to subunit size and relative abundance of several amino acid residues) from the reported amino acid composition of the pea chloroplastic isozyme (11) upon which Kwiatowski and Kaniuga based their comparison.

***In Vitro* Transcription/Translation.** The pSP64 vector containing the cDNA from pA2 was designated pSPA2. After linearization of pSPA2 with *Pvu* II, *in vitro* transcription yielded a transcript of ≈1100 nucleotides (Fig. 5). The transcript is of a size predicted from the size of the DNA insert (875 bp), together with the 180-bp *Eco*RI–*Pvu* II fragment and the 55-bp multiple cloning site of the vector. It was found to be ≈100 bases larger than the corresponding mRNA isolated from pea leaves (Fig. 5).

Cell-free translation of the pSPA2 *in vitro* transcript resulted in the formation of three polypeptides of 26, 23.5, and 20 kDa, respectively (Fig. 6, lane 1). The 23.5-kDa polypeptide was the predominant translation product, whereas the abundance of the other two varied from translation to translation. All three polypeptides were immunoprecipitable with anti-pea Cu/Zn-SOD antibodies (data not shown) and were absent from translations that did not contain the pSPA2 transcript (Fig. 6, lane 2). The pSPA2 construct contains only one translation start signal between the SP6 promoter and the 3′ end of the cDNA insert. It is possible, though, that the two secondary translation products are *in vitro* artifacts, resulting from initiation of translation at sites that would not be used *in vivo*.

Import of *in Vitro* Translation Products into Chloroplasts. To confirm that the cloned cDNA encoded the chloroplastic

FIG. 5. RNA blot hybridization of pea total RNA and the *in vitro* transcript of pSPA2. Hybridization was carried out with a ^{32}P-labeled *Eco*RI–*Hin*dIII fragment of plasmid pA2. Lane 1, *in vitro* transcript (20 ng) of pSPA2; lane 2, pea total RNA (20 μg). The sizes of molecular markers are given in kilobases. The arrowhead points to the Cu/Zn-SOD transcript.

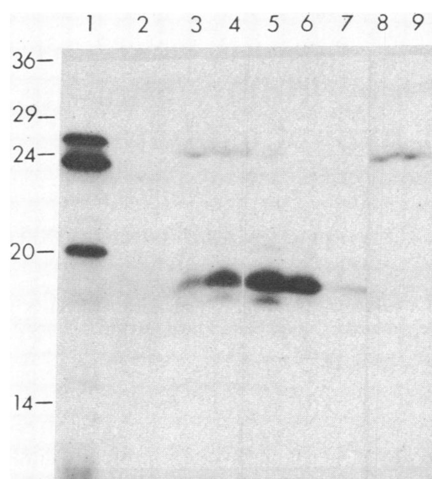

FIG. 6. Transport of *in vitro* translation products into isolated chloroplasts. Lane 1, 1 μl of cell-free translation product of the pSPA2 *in vitro* transcript; lane 2, cell-free translation (8 μl) without added RNA; lanes 3–5, import of the 23.5-kDa precursor polypeptide into chloroplasts over 5 (lane 3), 15 (lane 4), and 30 min (lane 5); lanes 6 and 7, prior to lysis, chloroplasts were resuspended in buffer containing 50 μg of thermolysin per ml, without (lane 6) or with (lane 7) 0.3% Triton X-100; lanes 8 and 9, transport assays carried out 30 min in darkness (lane 8) or 30 min in light in the presence of 50 nM nigericin (lane 9). Molecular masses are shown in kDa.

isozyme of pea Cu/Zn-SOD, *in vitro* transcription/translation products obtained from pSPA2 were incubated with intact, isolated pea chloroplasts. As illustrated in Fig. 6, the 23.5-kDa *in vitro* translation product decreased in abundance over a 30-min incubation with chloroplasts, whereas another polypeptide, migrating in the gel to the same position as the known pea chloroplastic SOD subunit, appeared. This 17.4-kDa polypeptide was absent from assays carried out either in the dark or in the presence of 50 nM nigericin; both conditions inhibit the formation of ATP, which is required for the transport of bound precursor polypeptides into chloroplasts (31). To ascertain that the newly appearing polypeptide was indeed sequestered within the chloroplasts, samples were subjected to a 30-min incubation in buffer containing 50 μg of thermolysin per ml in the presence or absence of 0.3% Triton X-100. Samples treated with protease alone contained the 17.4-kDa protein but not the 23.5-kDa precursor polypeptide, whereas protease and Triton X-100 treatment resulted in the degradation of both polypeptides. Thus, the newly formed protein was protected within the chloroplasts from the protease, whereas the precursor polypeptide, presumably bound to the outer chloroplast membrane, was not.

In summary, immunological screening of a pea leaf cDNA library resulted in the identification of a cDNA encoding the chloroplastic isozyme of pea Cu/Zn-SOD. The clone encodes a polypeptide composed of the mature SOD subunit and a 48-amino acid transit peptide. This precursor polypeptide is post-translationally processed upon import into isolated chloroplasts.

The deduced amino acid sequence of the mature pea chloroplastic Cu/Zn-SOD bears strong resemblance to amino acid sequences of other plant Cu/Zn-SODs. Although the greatest similarity is found between chloroplastic isozymes, nucleotide sequence homology between the cDNAs encoding the pea chloroplastic and the maize cytosolic isozymes indicates that the chloroplast SOD-encoding cDNA will be useful in identifying clones from the pea cDNA library that encode the cytosolic isozyme. Together, the two cDNAs can be used as mRNA-specific probes to analyze the transcriptional regulation of the two isozymes in pea, particularly in response to environmental conditions known to stimulate superoxide production in chloroplasts.

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1. Asada, K. & Takahashi, M. (1987) in *Photoinhibition*, eds. Kyle, D. J., Osmond, C. B. & Arntzen, C. J. (Elsevier Science, Amsterdam), pp. 227–287.
2. Steinman, H. M. (1982) in *Superoxide Dismutase*, ed. Oberley, L. W. (CRC, Boca Raton, FL), Vol. 1, pp. 11–68.
3. Jackson, C., Dench, J., Moore, A. L., Halliwell, B., Foyer, C. H. & Hall, D. O. (1978) *Eur. J. Biochem.* **91**, 339–344.
4. Salin, M. L. & Bridges, S. M. (1981) *Biochem. J.* **195**, 229–233.
5. White, J. A. & Scandalios, J. G. (1987) *Biochim. Biophys. Acta* **926**, 16–25.
6. Autor, A. P. (1982) *J. Biol. Chem.* **257**, 2713–2718.
7. Marres, C. A., Van Loon, A. P. G. M., Oudshoorn, P., Van Steeg, H., Grivell, L. A. & Slater, E. C. (1985) *Eur. J. Biochem.* **84**, 153–161.
8. Kitagawa, Y., Tsunasawa, S., Tanaka, N., Katsuki, Y., Sukiyama, F. & Asada, K. (1986) *J. Biochem.* **99**, 1289–1298.
9. Steffens, G. J., Michelson, A. M., Otting, F., Puget, K., Strassburger, W. & Flohe, L. (1986) *Biol. Chem. Hoppe-Seyler* **367**, 1007–1016.
10. Cannon, R. E., White, J. A. & Scandalios, J. G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 179–183.
11. Duke, M. V. & Salin, M. L. (1983) *Phytochemistry* **22**, 2369–2373.
12. Beauchamp, C. & Fridovich, I. (1971) *Anal. Biochem.* **44**, 276–287.
13. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
14. Smith, D. E. & Fisher, P. A. (1984) *J. Cell Biol.* **99**, 20–28.
15. Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203.
16. Gantt, J. S. & Key, J. L. (1986) *Mol. Gen. Genet.* **202**, 186–193.
17. Huynh, T., Young, R. & Davis, R. (1985) in *DNA Cloning: A Practical Approach*, ed. Glover, D. M. (IRL, Oxford), Vol. 1, pp. 49–78.
18. Davis, L. G., Dibner, M. D. & Battey, J. F. (1986) *Methods in Molecular Biology* (Elsevier, New York), pp. 158–160.
19. Hanahan, D. (1985) in *DNA Cloning: A Practical Approach*, ed. Glover, D. M. (IRL, Oxford), Vol. 1, pp. 109–135.
20. Sanger, F., Nicklen, S. & Coulson, R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
21. Hattori, M. & Sakaki, Y. (1986) *Anal. Biochem.* **152**, 232–238.
22. Birnboim, C. & Doly, J. (1979) *Nucleic Acids Res.* **7**, 1513–1523.
23. Bywater, M., Bywater, R. & Hellman, L. (1983) *Anal. Biochem.* **132**, 219–224.
24. Roberts, B. E. & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2330–2334.
25. Gantt, J. S. & Key, J. L. (1983) *Biochemistry* **22**, 4131–4139.
26. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
27. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
28. Orozco, E. M., Mullet, J. E., Hundley-Bodoin, L. & Chua, N.-H. (1986) *Methods Enzymol.* **118**, 232–253.
29. Pain, D. & Blobel, G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3288–3292.
30. Devereaux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395.
31. Schmidt, G. W. & Mishkind, M. L. (1986) *Annu. Rev. Biochem.* **55**, 879–912.
32. Lutke, H. A., Chow, K. C., Mickel, F. S., Moss, K. H., Kern, H. F. & Scheele, G. A. (1987) *EMBO J.* **6**, 43–48.
33. Elliston, K. & Messing, J. (1988) in *Architecture of Eukaryotic Genes*, ed. Kahl, G. (VCH, Weinheim, F.R.G.), pp. 21–56.
34. Steffens, G. J., Bannister, J. G., Bannister, W. H., Flohe, L., Gunzler, W. A., Kim, S.-M. A. & Otting, F. (1983) *Hoppe-Seyler's Z. Physiol. Chem.* **364**, 675–690.
35. Hjalmarsson, K., Marklund, S., Engstrom, Å. & Edlund, T. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6340–6344.
36. Kwiatowski, J. & Kaniuga, Z. (1986) *Biochim. Biophys. Acta* **874**, 99–115.