Hydroxypyruvate Reductase with a Carboxy-Terminal Targeting Signal to Microbodies is Expressed in Arabidopsis

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Five Arabidopsis EST cDNA clones of hydroxypyruvate reductase (HPR), a photosynthetic enzyme in leaf peroxisomes, were sequenced. Deduced amino acid sequences revealed that HPR in Arabidopsis contained the carboxy-terminal targeting signal to microbodies. Nucleotide sequence analysis showed that the cDNA with the longest insert contained an open reading frame of 1,158 bp which encoded a polypeptide with 386 amino acids with a calculated molecular mass of 42,251 Da. A Southern blot analysis suggested that the Arabidopsis HPR gene, like that of the pumpkin HPR gene, exists as a single copy. Two kinds of pumpkin HPR mRNA might be produced from a single gene by alternative splicing, but the structure of the genomic DNA indicated that the Arabidopsis HPR gene did not undergo alternative splicing. We detected a polypeptide with a molecular mass of 42 kDa in green leaves of Arabidopsis using an HPR-specific antibody. Immunoelectron microscopy revealed that Arabidopsis HPR protein was exclusively localized in leaf peroxisomes in green leaves. These results indicate that HPR is expressed in a form with a carboxy-terminal targeting signal to microbodies and is localized in microbodies in Arabidopsis, suggesting that the differences in the gene structure and the regulation of gene expression of HPR are probably due to species-specific differences in plants.

Key words: Alternative splicing — Arabidopsis thaliana — Hydroxypyruvate reductase (EC 1.1.1.29) — Leaf peroxisome — Targeting signal.

NADH-hydroxypyruvate reductase (HPR; EC 1.1.1.29), which is one of the leaf-peroxisomal enzymes, catalyzes the reduction of hydroxypyruvate to glyceraldehyde. The enzyme is known to be localized in leaf peroxisomes, which are specialized microbodies found in photosynthetic tissues, and they play a role in the glyceraldehyde pathway of photosynthesis in concert with enzymatic reactions in chloroplasts and mitochondria (Tolbert et al. 1968). HPR activity and transcripts are developmentally regulated and enhanced by light (Greener et al. 1989) and similar results have been observed for other leaf-peroxisomal enzymes such as glycolate oxidase (Tsugita et al. 1993). cDNAs for HPR have been cloned from pumpkin (Hayashi et al. 1996b), cucumber (Greener et al. 1989) and methylotrophs (Chistoserdova and Lidstrom 1994, Yoshida et al. 1994). In plants, HPR functions in the photosynthetic glyceraldehyde pathway, whereas in methylotrophs, it acts as an assimilatory enzyme of one-carbon components, such as methanol, in the serine pathway.

Previous work in our laboratory has shown that two kinds of cDNA clones for HPR (HPR1 and HPR2) were screened from the cDNA library in pumpkin green cotyledons (Hayashi et al. 1996b). The nucleotide sequences of these clones are identical except for three single-nucleotide substitutions and for the region encoding the carboxy terminus. The deduced HPR1 protein contains the carboxy-terminal tripeptide of Ser-Lys-Leu, which is known as a targeting signal to microbodies (Gould et al. 1988, 1989) but the deduced HPR2 protein does not. These results suggested that HPR1 protein is transported into leaf peroxisomes and HPR2 protein remains in the cytosol. Moreover, analysis of the structure of an alternative splicing suggests that alternative splicing gives rise to two kinds of HPR mRNA. We have detected two polypeptides in homogenates from pumpkin leaves (see results), and have confirmed that one of them is localized in leaf peroxisomes and the other is localized in the cytosol of green leaves (Mano, Hayashi, Kondo and Nishimura. unpublished).

Alternative splicing is a well-known post-transcriptional regulatory mechanism in eukaryotic organisms. It has been reported that the synthesis of several enzymes are controlled by alternative splicing (Corri et al. 1994, Görlich et al. 1995, Hayashi et al. 1996b, Kopriva et al. 1995, Sugiyama et al. 1996, Tamaoki et al. 1995, Theil et al. 1992). Recent analysis from mammalian showed the presence of SR proteins, which are regarded as candidates for alternative splicing factor (Gontarek and Derse 1996, Scream et al. 1995). Lopato et al. (1996) demonstrated that plants also possessed SR proteins. These SR proteins from carrot, tobacco and Arabidopsis were active in an alternative splicing assay in vitro. Moreover, the existence of exonic splicing enhancers, which are sequences to which SR proteins are able to bind, has been demonstrated (Gontarek and...
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(Murray and Thompson 1980). The reaction mixture contained 1 unit of Ampli Taq DNA polymerase (Perkin Elmer Japan, Chiba, Japan), an upstream primer (ATHPR1; 5'-GCATGTCACATCAGACCGCTA-3'), a downstream primer (ATHPR2; 5'-GACAATACAAAGTTGAC-3') and an appropriate buffer in a total volume of 50 µl. Each reaction was at 94°C for 45 s, 55°C for 45 s and 72°C for 45 s. The DNA fragment was subcloned into a T-vector prepared using pBluescript KS+ as described in a previous report (Marchuk et al. 1990).

Southern blot analysis—Total DNA (1 µg) was digested with BglII and XbaI. The products were fractionated on a 0.8% agarose gel, transferred to a Zeta-Probe blotting membrane (BIORAD, CA, U.S.A.) by the capillary method and fixed by exposure to UV-light (Funa-UV-Linker, model FS-800; Funakoshi, Tokyo, Japan). The fragment corresponding to Arabidopsis HPR EST clone (113J10T7) was labeled with [³²P]dCTP (Amersham Japan) using a BeCBest labeling kit (Takara Shuzo, Kyoto, Japan). The membrane was hybridized in 50% formamide, 0.12 M sodium phosphate (pH 7.2), 0.25 M sodium chloride, 7% SDS and 1 mM EDTA (pH 8.0) with 1.0 × 10⁴ cpm ml⁻¹ of radiolabeled DNA probe for 18 h at 42°C. The membrane was washed at 42°C in 2 × SSC plus 0.1% SDS for 15 min, in 0.2 × SSC plus 0.1% SDS for 15 min, in 0.1 × SSC plus 0.1% SDS for 15 min. The membrane was used to expose X-ray film.

Immunoblot analysis—The leaves of Arabidopsis plants and pumpkin cotyledons at the 21-old-day stage were homogenized with the extraction buffer (100 mM Tris-HCl, pH 6.8, 1 mM EDTA, pH 8.0) with 1.0 × 10⁴ cpm ml⁻¹ of radiolabeled DNA probe for 18 h at 42°C. The membrane was washed at 42°C in 2 × SSC plus 0.1% SDS for 15 min, in 0.2 × SSC plus 0.1% SDS for 15 min, in 0.1 × SSC plus 0.1% SDS for 15 min. The protein content of each extract was estimated by using a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo, Japan) with bovine gamma albumin as a standard protein. Five or ten µg of total protein was separated by SDS-PAGE on a 7.5% polyacrylamide gel as described by Laemmli (1970) and transferred to a nylon membrane (Schleicher & Schuell, Dassel, Germany) in a semidry electrophoretic system. Immunologic reactions were detected by monitoring the activity of horseradish peroxidase antibodies against rabbit IgG (ECL system; Amersham, Japan).

Immunoelectron microscopy—Arabidopsis leaves at the 21-old-day stage were fixed, dehydrated and embedded in LR white resin (London Resin, U.K.) as described previously (Nishimura et al. 1993). Ultra-thin sections were cut on a Reichert ultramicrotome (Leica, Heidelberg, Germany) with a diamond knife and mounted on uncoated nickel grids. The protein A-gold labeling procedure was essentially the same as that described by Nishimura et al. (1993). Ultra-thin sections were incubated at room temperature for 1 h with a solution of antiserum against HPR diluted 1 : 2,000 and then with a 50-fold diluted suspension of protein A-gold (Amersham Japan) at room temperature for 30 min. The sections were examined with a transmission electron microscope (1200EX; JOEL, Tokyo, Japan) at 80 kV.

Results and Discussion

Full-length sequence of Arabidopsis HPR cDNA—An EST bank at ABRC at Ohio State University was screened for HPR cDNA in Arabidopsis using the amino acid sequence of pumpkin HPR1 protein. Five cDNA clones showed high similarities to pumpkin HPR1. The stock numbers of these clones were 60A7T7 (accession No. T41584), 135P15T7 (accession No. T75991), 113J10T7 (accession No. T42460), 183L19T7 (accession No. H37101) and
133M20T7 (accession No. T45536). The complete nucleotide sequence of 113J10T7, which contained the longest insert, was determined. As shown in Figure 1, this cDNA consists of 1,428 bp and contains a 1,158-bp open reading frame that encodes a polypeptide with 386 amino acids with a total molecular mass of 42,251 Da. The deduced sequence beginning with the first ATG at nucleotide 81 showed high similarities to HPRs from other sources such as pumpkin (Hayashi et al. 1996b), cucumber (Greenler et al. 1989) and methylotrophs (Chistoserdova and Lidstrom 1994, Yoshida et al. 1994). The identities with amino acid sequences of HPR for other plants are 88% for pumpkin HPR1, 87% for pumpkin HPR2 and 87% for cucumber. The carboxy-terminal sequence was Ser-Lys-Leu, which is known to be one of the targeting signals to microbodies (Gould et al. 1988, 1989).

**Nucleotide sequences around the carboxy terminus of Arabidopsis EST clones**—The carboxy-terminal targeting signals to microbodies are characterized well in glyoxysomal- and peroxisomal-enzymes (Gould et al. 1988, 1989, 1996).

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**Fig. 1** Nucleotide and deduced amino acid sequences of Arabidopsis HPR DNA. The complete nucleotide sequence of 113J10T7 was determined. The nucleotide sequence of the putative coding region is shown in uppercase letters and the 5'- and 3'-noncoding regions are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence, starting at the first in-frame methionine residue and ending at the first stop codon, indicated by an asterisk. The three carboxy-terminal amino acids that are known as a microbody-targeting signal are boxed. Arrows indicate primers, ATHPR1 and ATHPR2, for PCR, respectively.
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Hayashi et al. 1996a, Mano et al. 1996, Mori et al. 1991, Olsen et al. 1993, Tsugeki et al. 1993, Volkita 1991). But another HPR protein in pumpkin (HPR2) does not have this signal. We determined the nucleotide sequences encoding the region around the carboxy terminus of other clones in order to identify whether or not these sequences contained the targeting signal to microbodies. As shown in Figure 2, the nucleotide sequences of all cDNAs were identical, although the lengths and positions of the poly-(A) tail were different. All deduced amino acid sequences contained the Ser-Lys-Leu sequence at the carboxy terminus and none of the HPRs were found to lack the targeting signal, as occurs in pumpkin HPR2 cDNA.

**Genomic sequence and putative amino acid sequence of Arabidopsis HPR.—** We could not exclude the possibility of the presence of another type of cDNA in *Arabidopsis* such as pumpkin HPR2 cDNA and cucumber HPR cDNA (Greenler et al. 1989), which lacked the Ser-Lys-Leu sequence at their carboxy terminus, because it might not be registered in the EST bank. We have reported that HPR without the targeting signal may be produced from one gene by alternative splicing since the genomic structure contains two pairs of GT-AG doublets (Hayashi et al. 1996b), so two kinds of mRNA are produced depending on the way in which the exons are spliced. It is assumed that the longer nucleotide sequence flanked by GT-AG is removed as it is in the case of HPR1. HPR2 is produced when the shorter intron, which does not contain the first 17 bp with a stop codon, is eliminated. There is a similar structure around intron XII of cucumber genomic DNA (Hayashi et al. 1996b).

![Alignment of nucleotide sequences encoding the carboxy-terminal amino acids of *Arabidopsis* EST clones. All clones were obtained from ABRC. The nucleotide sequence of the coding region is shown in uppercase letters and the 3’-noncoding region is shown in lowercase letters. The deduced amino acid sequence around the carboxy terminus is presented in the single-letter code over the nucleotide sequence. Identical nucleotides are indicated by an asterisk under the sequence. The stock number of each clone is shown on the left.](image-url)
Since *Arabidopsis* HPR does not seem to be produced by alternative splicing, does another HPR gene exist in *Arabidopsis*? Givan and Kleczkowski (1992) detected the activities and polypeptides of HPR in leaf peroxisomes and the cytosol of barley leaves. Judging from the difference in subunit molecular masses between HPR1 and HPR2 in barley, they seem to be derived from different genes, whereas Greener et al. (1989) reported that a single gene for HPR is present per haploid genome in cucumber, and we confirmed that pumpkin HPR was also a single-copy gene (Mano, Hayashi, Kondo and Nishimura. unpublished). Therefore, we carried out a Southern blot analysis. DNA from leaves of *Arabidopsis* was isolated, digested with *BglII* and *XbaI*, none of which cuts *Arabidopsis* cDNA (113J10T7), and subjected to Southern blot hybridization using *Arabidopsis* HPR cDNA as a probe. As shown in Figure 4, HPR cDNA hybridized with a single fragment. In addition, only one kind of genomic sequence was obtained when we amplified the genomic DNA (Fig. 3). These findings indicate that HPR in *Arabidopsis* exists as a single-copy gene, as it does not occur.

*Arabidopsis* HPR is encoded by a single copy gene—

**Fig. 3** Structural relationships around the carboxy termini of HPR from *Arabidopsis* and pumpkin. (A) The nucleotide sequence of the intron in this region of *Arabidopsis* HPR gene is shown. The nucleotide sequence of the exon is shown in uppercase letters and the intron and 3′-noncoding region are shown in lowercase letters. The deduced amino acid sequence is presented in the single-letter code under the nucleotide sequence. Asterisk indicates the stop codon and boxes represent the consensus sequences found in the beginning (GT) and the end (AG) of an intron. (B), (C) Schematic representation between the proteins deduced from *Arabidopsis* mRNA (B) and pumpkin mRNAs (C) and each gene. The hatched boxes correspond to exons and the open boxes correspond to introns. The consensus sequences corresponding to the splice donor and acceptor sites and the carboxy-terminal amino acids are shown. Asterisk shows the stop codon.
in pumpkin and cucumber.

**Antibodies against HPR cross-react with a 42 kDa polypeptide**—Immunoblotting was carried out using antibodies against spinach HPR. Total proteins prepared from *Arabidopsis* and pumpkin, as a reference, were subjected to SDS-PAGE. A unique polypeptide with a molecular mass of 42 kDa was recognized by the antibodies against spinach HPR in the total *Arabidopsis* extract (Fig. 5, lane 1 and 2). This is in good agreement with the molecular mass calculated from the deduced amino acids. In the case of total protein from pumpkin, two polypeptides were detected (Fig. 5, lane 3). We previously reported the presence of two kinds of HPR cDNA in green pumpkin cotyledons, which were designated HPR1 and HPR2 (Hayashi et al. 1996b). The molecular masses of the HPR1 and HPR2 proteins are calculated to be 42,305 Da and 41,709 Da from their deduced amino acids, respectively. This unique band in *Arabidopsis* did not split into two bands under the same electrophoretic condition, even though the volume of total protein was reduced (Fig. 5, lane 1). These data indicated that HPR in *Arabidopsis* is only expressed as a 42 kDa polypeptide. Based on these data, we postulate that *Arabidopsis* has one kind of HPR enzyme (from a single gene) and pumpkin has two kinds of HPR enzyme produced by alternative splicing. These results indicate that the structure of the HPR gene varies with plant species and that the regulation of expression of the HPR gene may be different.

*Arabidopsis* HPR *is exclusively localized in leaf peroxisomes*—The localization of HPR was investigated using immunoelectron microscopy. Gold particles for HPR were exclusively detected in leaf peroxisomes but not in other organelles such as mitochondria, chloroplasts and vacuoles (Fig. 6). This electron micrograph clearly shows that HPR is transported into leaf peroxisomes in *Arabidopsis*. This is in agreement with the result that *Arabidopsis* HPR is detected as one kind of polypeptide and that the expression of HPR is not regulated by alternative splicing. Further analysis will be required to understand the detailed mechanism of alternative splicing in higher plants.

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