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Complete structure of the gene for phospho*enol*pyruvate carboxylase from maize

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Phospho*enol*pyruvate carboxylase is a key enzyme in photosynthesis in some plants that exploit the C_4 photosynthetic pathway for the fixation of CO_2. We cloned the gene for this enzyme from maize genomic libraries and analyzed its complete primary structure. The sequence of the cloned gene spans 6781 bp and consists of 10 exons and 9 introns. The site of initiation of transcription is located 84 nucleotides upstream from the first nucleotide of the initiation codon (position −84), as determined by the method of primer-extension analysis. The analysis suggests that there is another initiation site located at position −81. The 5′-flanking region of the gene lacks typical TATA and CCAAT elements in the anticipated regions, but there is a TATA-similar sequence (TATTT) around the −30 regions as well as sequence homologous to the Sp-1 protein-binding site (CCGCCC). Six long, direct repeated sequences and a light-responsive element are also present in the 5′-flanking region. The results of Southern blot analysis indicated that the phospho*enol*pyruvate carboxylase gene exists as a small multi-gene family, but the enzyme that is expressed at high levels in green leaves and is involved in C_4 photosynthesis is encoded by a single-copy gene in the maize genome.

Phospho*enol*pyruvate carboxylase catalyzes the fixation of atmospheric CO_2 on phospho*enol*pyruvate (*P*-pyruvate) to form oxaloacetate in C_4 plants and crassulacean acid metabolism plants; it plays an important role in the C_4 photosynthetic pathway (reviewed in [1, 2]). C_4 plants show higher rates of photosynthesis than C_3 plants at high light intensities and high temperatures, because CO_2 is concentrated in bundle sheath cells by the C_4 metabolic pathway; the oxygenase reaction of ribulose-1,5-bisphosphate carboxylase is suppressed or prevented so that photorespiration is negligible [3]. For these reasons *P*-pyruvate carboxylase is regarded as an important target in efforts to improve the productivity of C_3 plants such as rice, wheat, and bean. The enzyme from the C_4 plant, maize, is composed of four identical subunits (of molecular mass 100 kDa) encoded by nuclear DNA [4]. Recently, Izui and his colleagues [5, 6] reported the structure of *P*-pyruvate carboxylase cDNA from maize and deduced that the maize enzyme is composed of 970 amino acid residues with a molecular mass of 109408 Da. C_3 plants also have *P*-pyruvate carboxylase enzymes and their structures are quite similar to those from C_4 plants [7]. However, the expression of the enzyme in C_3 and C_4 plants is very different. The C_4 enzymes are localized exclusively in the cytoplasm of mesophyll cells, and a low level is found in bundle sheath cells [8, 9]. By contrast, the enzyme in C_3 plants is expressed not only in mesophyll cells but also in various other tissues, such as root [10], guard cell [11], and epidermis [12]. The differential localization of the enzyme is regulated at the transcriptional level. The expression of the C_4 gene is also regulated by light, which stimulates an increase in the steady-state level of *P*-pyruvate carboxylase mRNA [9, 13, 14].

We have been conducting biochemical and molecular-biological comparisons of C_4 photosynthetic enzymes between C_3 and C_4 plants because these enzymes can be used in the study of changes in gene expression during the evolution of C_3 to C_4 photosynthesis [7, 15, 16]. The comparison of genes for C_4 photosynthetic enzymes between C_3 and C_4 plants provides a useful method for elucidating the way in which C_4 plants have gained the unique properties associated with the regulation of expression of these enzymes. However, there are no published reports about the structures of genes for C_4 photosynthetic enzymes. In this paper we report the isolation and structural characterization of a cloned genomic sequence that contains the complete *P*-pyruvate carboxylase gene from maize. We discuss the putative regulatory sequence in the 5′-flanking region of the gene.

EXPERIMENTAL PROCEDURES

Maize (*Zea mays* L. cv. Golden Cross Bantam) was planted in vermiculite and grown in a growth chamber at 30 °C for 4 days under dark or light (20 klx) conditions. Genomic DNA was isolated from etiolated leaves as previously described [17]. Poly(A)-rich RNA was prepared from green leaves as described elsewhere [18].

Cloning and screening of the genomic DNA for *P*-pyruvate carboxylase were performed as follows. Genomic DNA was completely digested with *Xba*I and fractionated by centrifugation on 10–40% sucrose density gradients. A 9-kb DNA fragment containing the *P*-pyruvate carboxylase gene was recovered and ligated to the *Xba*I arms of the phage *λong* C (Stratagene). The ligated DNA was packaged *in vitro* [19]. The phage plaques were screened by plaque hybridization using a cDNA clone for the maize enzyme as a probe [16].

The nucleotide sequence of the genomic clone was determined by the dideoxynucleotide chain-termination method [20]. Primer-extension analysis was carried out according to the method of Ishii et al. [21] using a synthetic 17-residue

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Abbreviation. *P*-pyruvate, phospho*enol*pyruvate.

Enzyme. Phospho*enol*pyruvate carboxylase (EC 4.1.1.31).

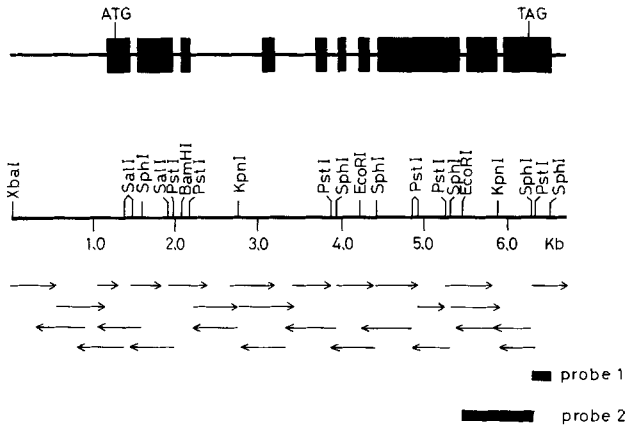

Fig. 1. *Structure of the* P-*pyruvate carboxylase gene and sequencing strategy.* Locations of exon and intron regions are presented as boxes and lines between boxes, respectively. ATG and TAG indicate the locations of the initiation and the termination codons of the *P*-pyruvate carboxylase gene, respectively. A restriction map of the gene is also presented. The directions and lengths of the sequences determined are shown by horizontal arrows. Probes 1 and 2 (0.3-kb *Sph*I and 0.8-kb *Eco*RI–*Sph*I fragments, respectively), used for Southern blot analysis, are shown by thick lines

oligonucleotide of which the 5′ end was labeled with ^{32}P. Southern blot analysis was performed as described elsewhere [19].

RESULTS AND DISCUSSION

Isolation and characterization of the P-*pyruvate carboxylase gene from maize*

It has been reported that, in maize plants, there are two types of *P*-pyruvate carboxylase: one is involved with the C_4 photosynthetic pathway (C_4 type) and the other is not involved with that pathway (C_3 type); their characteristics are different but their nucleotide sequences are similar and they cross-hybridize [1, 2, 7, 22].

Thus, we used the 3′-noncoding region of cDNA for C_4-type *P*-pyruvate carboxylase as a probe to screen a maize genomic library, in order to obtain the C_4-type gene that is expressed in green leaves. Three clones were isolated from 5×10^5 recombinant phages and analyzed by restriction endonuclease mapping and Southern blotting. These clones also hybridized with the 5′-noncoding region of the cDNA and had the same restriction maps. These results indicate that the clones are identical and contain the complete region of the cDNA clone. One of these clones was chosen for further analysis.

Fig. 1 shows the restriction map and the strategy for sequencing of the cloned gene. The analyzed sequence comprises 2910 bp which cover the coding region (970 amino acid residues, including the initiator methionine), all introns and the 5′- and 3′-flanking regions, for a total of 6781 bp (Fig. 2). The sequence of the coding region is quite similar to the previously reported cDNA sequence for maize *P*-pyruvate carboxylase [5, 6]. Both coding regions are the same size (2910 bp), and only 37 nucleotide differences are found. These nucleotide differences correspond to a total of 7 amino acid exchanges out of a total of 970 amino acid residues. In the 5′- and 3′-noncoding regions (64 bp and 219 bp, respectively), both sequences are also quite similar to each other, only two and three nucleotides differences, respectively, being found. This high degree of similarity indicates that the isolated genomic clone corresponds to the gene that encodes the C_4-type *P*-pyruvate carboxylase. The results also indicate that the gene is an active gene transcribed in the green leaves of maize and is not a pseudogene. The structural discrepancies between the genomic DNA and the cDNA clones may be caused by the difference in maize cultivars. The cultivar we used to obtain the genomic clone is Golden Cross Bantam and the cDNA clone was obtained from the cultivar, indentata Sturt [5].

The *P*-pyruvate carboxylase gene contains ten exons which are separated by nine introns (Figs 1 and 2). The sizes of both the exon and intron regions vary considerably: exons range between 85 and 999 bp, while introns range between 92 and 897 bp in length. These intron sequences account for 39% of the length of the primary transcript. The first exon contains 84 bp of 5′-untranslated region (see below) which does not contain any false translation initiation sites. This result corresponds to the observation that the first ATG codon on the processed mRNA acts as a site for initiation of translation in a majority of plant genes. The sequence around the translation initiation site, CGCCATGGC, does not correspond well to the reported consensus sequence for plants, AACAATGGC [23], but it does resemble the animal consensus sequences, C(A/G)CCATGGC [24]. Exon 10 consists of 294 bp of coding region and 270 bp of 3′-untranslated region which contains two putative polyadenylation signals, AATAAA or AATAAT, underlined in Fig. 2. In fact, it has been shown by the sequencing of 3′-untranslated regions of the cDNA clones that the poly(A) tract is attached at multiple sites *in vivo* [6].

In the all introns, typical GT/AG donor-acceptor sites are present, and the sequences surrounding these splice junctions are similar to the plant consensus sequence but are not identical (Table 1). The splice junction of the plant consensus sequence is similar to that of animal introns except that the polypyrimidine stretch at the 3′ splice junction is less pronounced in plant introns [25]. In the introns of the *P*-pyruvate carboxylase gene, stretches of five or more consecutive pyrimidine residues at the 3′ splice junctions are not found. This result indicates that the structures of the introns of this gene correspond to the consensus structure of plant introns but not to that of animal introns. Recently, Hanley and Schuler [26] reported that the purine- or pyrimidine-richness of sequences upstream from the 3′ splice site differs significantly between introns in monocotyledons and dicotyledons. According to their classification, introns 2, 3, 8 and 9 are classified as pyrimidine-rich, while introns 1, 4, 5, 6 and 7 are classified as being of mixed type. The purine-rich type of intron is not found among *P*-pyruvate carboxylase gene introns. This result is consistent with the evaluation of Hanley and Schuler. They observed that a higher proportion of dicotyledon introns is of the purine-rich type while a higher proportion of monocotyledon introns is pyrimidine-rich. However, they also evaluated the differences between the 5′ splice sites in dicotyledon and monocotyledon introns; we were unable to find such nucleotide preferences as they propose in the introns of the *P*-pyruvate carboxylase gene.

Determination of the site of initiation of transcription

The transcription initiation site was determined by primer-extension analysis. A synthetic 17-residue oligonucleotide corresponding to the anti-sense strand starting two bases upstream from the translation initiation site (5′-GCGCGGCG-

Fig. 2. *The complete nucleotide sequence of the* P*-pyruvate carboxylase gene and flanking regions.* The numbers on the right refer to the nucleotide number starting with the most probable site of initiation of transcription as number 1 (marked with an asterisk on top). Sequences homologous to the Sp1-binding site and polyadenylation signals are underlined. Boxes indicate the sequences that are similar to the TATA box and light-responsive elements. Six direct repeated sequences are indicated by horizontal arrows. Arrowheads indicate the poly(A) addition sites reported by Yanagisawa et al. [9]. The initiation and termination codons are shaded. The coding region is represented by capital letters and other regions by lower-case letters

Table 1. *Sequence of exon-intron splice junctions in the* P*-pyruvate carboxylase gene*
Capital letters represent exon sequences and lower-case letters represent intron sequences. Consensus indicates the consensus sequence in genes of monocotyledon plants, taken from Hanley and Schuler [26]. In the consensus sequence, n represents any nucleotide

ATTT	gtaactaac	-	IVS 1	-	ctgtaatgcatgcag	GTCC
CCAG	gtatatatt	-	IVS 2	-	gtcttgtcgctgcag	GATC
AGAG	gtacgtaca	-	IVS 3	-	cttgggtgttcacag	ATCC
GATG	gtacatttc	-	IVS 4	-	tctatcgctatatag	GAAA
TGAG	gtactgtac	-	IVS 5	-	ctgatatatctgtag	CTCT
ATAG	gtaaccaca	-	IVS 6	-	tatttgctggcgcag	AATT
AGAG	gtaaatatc	-	IVS 7	-	cgtgcatgcatgcag	TTCC
ATCG	gtatgctgc	-	IVS 8	-	gtcactcactatcag	GCTA
CCAG	gtaccaaaa	-	IVS 9	-	ctccattttgtgcag	ATCG
A CAG G	gta$^{a}_{t}$$^{tttt}_{g}$cacn		-CONSENSUS-		ccgacacgnn$^{ttttttttt}_{n}$^{g}cag	

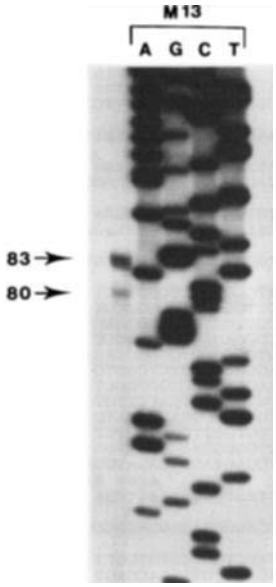

Fig. 3. *Mapping of the site of initiation of transcription by primer-extension analysis.* The synthetic anti-sense oligonucleotide corresponding to positions 72–83 was used as primer DNA. The probe labeled with [α-^{32}P]ATP was hybridized to poly(A)-rich RNA (20 µg) from green maize leaves and elongated by a reverse transcriptase. The extension products were analyzed in a 10% sequencing gel. The sequence of M13 mp18 was used to generate nucleotide size markers. Numbers on the left indicate the sizes in nucleotides of the extension products

GGAAGCTAA-3′) was used as a primer. The extension products were electrophoresed on a sequencing gel in parallel with a set of sequencing reactions of M13 DNA (Fig. 3). The main band was detected at a position that corresponded to fragments of 83 bases in length. This result indicates that the cap site is 84 nucleotides upstream from the first residue of the initiation codon. The sequence around the site, TTGATCA, is homologous with the consensus cap sequence of plant genes [23]. The 5′-untranslated region of the longest *P*-pyruvate carboxylase cDNA is 82 bp in length [6], confirming the result of the primer-extension analysis. An additional band was detected corresponding to a length of 80 bp. The sequence around the putative cap site, ATCACCA, is also homologous with the plant consensus cap sequence. Furthermore, another cDNA clone containing a 78-bp 5′-untranslated region has been obtained [6], suggesting that another cap site is located at the residue situated 81 nucleotides upstream from the first residue of the initiation codon.

Characterization of the 5′-flanking region of the gene

The expression of the C_4-type *P*-pyruvate carboxylase gene in maize is strictly limited to mesophyll cells of green leaves under light conditions [8, 9]. This is a specific characteristic of a C_4-type *P*-pyruvate carboxylase gene and not of a C_3-type one. Actually, these enzymes in C_3 plants can be detected in etiolated leaves [14] and in roots [10], and the structure of these enzymes is similar to that of C_4-type enzymes [7]. All available evidence suggests that C_4 plants have arisen from C_3 plants and that this transition has occurred polyphyletically many times during the course of evolution [27–29]. Therefore, it is possible that the unique properties of the C_4 *P*-pyruvate carboxylase gene have been added to those of the C_3 enzyme gene during the evolution of C_4 plants from C_3 plants. With this possibility in mind, we searched for the existence of some element(s) in the 5′-flanking region of the C_4 *P*-pyruvate carboxylase gene that might be involved in the unique mode of expression of this gene in C_4 plants.

The genomic clone contains 1212 bp of 5′-flanking sequence. It is well known that many genes of higher eukaryotes, including plants, have several characteristic sequence elements in their 5′-flanking regions [30], such as TATA and CCAAT (see Table 2). In the 5′-flanking sequence of *P*-pyruvate carboxylase no typical TATA element is found around −30 position but a similar sequence, TATTT, is found at positions −28 to −24. No such typical CCAAT element or similar sequence is found around position −80. Such an element is located further upstream at positions −365 to −369, but whether this CCAAT element is functional remains to be determined. Another characteristic sequence, CCGCCC, is found twice: at positions −47 to −52 and −79 to −84. The sequence is also found in the first intron at positions 282–287. The hexanucleotide sequence is known as the binding site for SV40 Sp1 protein [31]. A consensus sequence, (G/T)-GGGCGG(G/A)(G/A)(C/T), or its inverted form has been proposed to be the best Sp1-binding sequence [31]. The element in the *P*-pyruvate carboxylase gene do not match this consensus sequence, but the elements which presumably act as the promoter elements of several animal genes do not always conform to the consensus sequence [31]. There have been no studies to ascertain whether the hexanucleotide sequence, or any other promoter elements found in animal genes, has a promoter activity in plant cells. However, some plant genes include sequences similar to animal promoter elements, i.e. the SV40 enhancer core sequence, in putative enhancer regions [32, 33]. Thus, the hexanucleotide sequences in the *P*-pyruvate carboxylase gene may have promoter activity in maize cells. An unusual feature of the 5′-flanking region is that there are six long, direct repeat sequences (Table 2). These six direct repeat sequences contain many cytidine

Table 2. *Putative regulatory elements found in the* P. *pyruvate carboxylase gene*
The repeated sequences are aligned to give the most overlap and hyphens indicate gaps that are necessary to align the sequences

Element	Position	Sequence
TATA box	-24 to -28	TATTT
CCAAT box	-367 to -371	CCAAT
Sp-1 binding site	-80 to -85	CCGCCC
	-48 to -53	CCGCCC
	275 to 280(intron 1)	CCGCCG
	281 to 286(intron 1)	CCGCCC
light-responsive element	-653 to -661	CCTTATCCT
direct repeated sequence	-536 to -550	CCCTCAACCACATCCTGC
	-510 to -527	GACACCCTCG-CCACATCC
	-453 to -470	GACGCCCTCT-CCACATCCTGC
	-378 to -395	GACGCCCTCT-CCACATCCTGC
	-201 to -214	CCCTCT-CCACATCC
	-30 to -39	CT-CCCCATCC

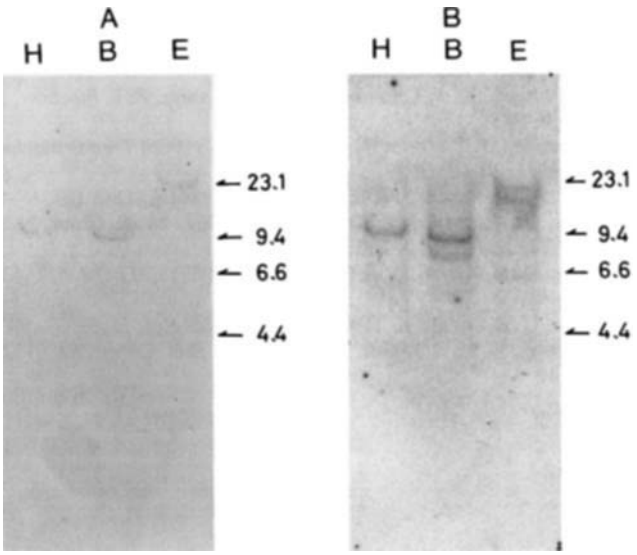

Fig. 4. *Southern blot hybridization of genomic DNA.* Maize genomic DNA (10 μg) was digested with *Hin*dIII (H), *Bam*HI (B), and *Eco*RI (E), and electrophoresed on a 0.7% agarose gel. Fractionated DNA was denatured, transferred to nitrocellulose, and hybridized to ^{32}P-labeled probes. (A) Probe 1 (see Fig. 1) containing the 3′ non-coding region was used. (B) Probe 2, containing the C-terminal side of the *P*-pyruvate carboxylase coding region. The length (in kb) of markers is shown at the right

residues (more than 50%) and are located at positions between −548 and −30. It has been suggested, in a gene for the small subunit of a C_3-type carboxylase enzyme (ribulose-bisphosphate carboxylase), that direct repeat sequences in the 5′-flanking region are involved in the regulation of the expression of the enzyme in C_3 mesophyll cells [34]. It is possible that the six direct repeat sequences in the *P*-pyruvate carboxylase gene may have a function(s) that is involved with the expression of the enzyme in maize mesophyll cells. Recently, Grob and Stüber [35] reported the existence of a common, light-responsive element in 5′-flanking regions of phytochrome-dependent, light-inducible plant genes. The *P*-pyruvate carboxylase gene has a similar sequence, CCTTATCCT, at positions −651 to −659; it is possible that this sequence plays a role in the control of the expression of the *P*-pyruvate carboxylase gene by light.

Genomic Southern hybridization

Southern hybridization was carried out to investigate the genomic organization of the *P*-pyruvate carboxylase gene and to estimate its copy number. High-molecular-mass genomic DNA was digested with *Eco*RI, *Bam*HI and *Hin*dIII, and then hybridized. When probed with the *Sph*I fragment which includes only the 3′ non-coding region (probe 1 in Fig. 1), one fragment hybridized with the probe in each lane (Fig. 4A). Comparison of the sequences of 3′ non-coding regions between *P*-pyruvate carboxylase cDNAs and the genomic clone indicates that the structure of the 3′ non-coding region of the C_4-type *P*-pyruvate carboxylase gene is well conserved in different cultivars of maize (see above). Yanagisawa et al. [6] analyzed five independent cDNA clones from green maize leaves and reported that their sequences are essentially the same except for their 3′-terminal length heterogeneity due to variation in the attachment sites for their poly(A) tracts. We also sequenced the 3′ non-coding regions of several *P*-pyruvate carboxylase cDNAs from green maize leaves and found that their sequences are the same and quite similar to the reported sequence (data not shown). These results indicate that the 3′ non-coding region of C_4-type *P*-pyruvate carboxylase genes is well conserved. Thus, the result of Southern hybridization analysis with the 3′ non-coding region of the genomic clone suggests that C_4-type enzymes is encoded by a single-copy gene in the maize genome. However, when probed with another fragment (*Eco*RI − *Sph*I fragment) which in-

cluded the C-terminal side of the coding region (probe 2 in Fig. 1), three *Eco*RI fragments, two *Bam*HI fragments, and one *Hin*dIII fragment were found to hybridize (Fig. 4B). Probe 2 is found adjacent to probe 1 and the two probes have no sites for cleavage by *Eco*RI, *Bam*HI and *Hin*dIII. Thus, probe 2 hybridizes with the same bands as those hybridized with probe 1; in fact, these three bands were detected in the digests. Additional bands not hybridized with probe 1 were found in the *Eco*RI and *Bam*HI digests. Harpster and Taylor [36] reported that *P*-pyruvate carboxylase cDNA from green maize leaves (C_4 type) hybridizes with *P*-pyruvate carboxylase mRNA from roots or etiolated leaves (C_3 type), but the two types of enzyme have different structures and the average difference in sequence is 7% between the two types. The divergence in sequence in the untranslated region is much higher than that in the coding region. Therefore, the bands hybridized with probe 2 but not with probe 1 may encode the coding region of C_3-type *P*-pyruvate carboxylase. However, further studies are required to elucidate whether the bands actually correspond to the C_3-type gene(s) expressed in the root or the etiolated leaf.

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