RNA editing is an important post-transcriptional process in chloroplasts and is thought to be functionally significant. Here we show a requirement of RNA editing for a functional enzyme. In peas, acetyl-CoA carboxylase (ACCase), a key enzyme of fatty acid synthesis, is composed of biotin carboxylase with the biotin carboxyl carrier protein and carboxyltransferase (CT). CT is composed of the nuclear-encoded $\alpha$ polypeptide and the chloroplast-encoded $\beta$ polypeptide in peas. One nucleotide of the $\beta$ polypeptide mRNA, which is edited in pea chloroplasts, converts the serine codon to the leucine codon. We show that this RNA editing is required for functional CT by comparing the unedited and edited recombinant enzymes. In plants not having a leucine codon at the same position, editing was shown to take place so as to create the leucine codon, indicating that editing is needed for in vitro CT activity and therefore for ACCase. To our knowledge, ACCase is an essential enzyme, suggesting that the chloroplast RNA editing is necessary for these plants.

RNA editing is one of the most interesting and universal RNA-processing mechanisms known to affect gene regulation. This process has been detected in a variety of organisms, such as trypanosomes and viruses (1, 2). In mammalian systems, both edited and unedited proteins are functional and have distinct properties (3–6). For example, the edited and unedited subunits of the glutamate receptor give rise to complex differing in calcium permeability (5). In chloroplasts, RNA editing is a widespread processing event, creating start and stop codons and most frequently altering coding sequences (7–11). To date, a number of RNA editing sites have been identified in chloroplasts with completely sequenced genomes, for example 26 plastids with completely sequenced genomes, for example 26

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§ The abbreviations used are: ACCase, acetyl-CoA carboxylase; CT, carboxyltransferase; PAGE, polyacrylamide gel electrophoresis.

**EXPERIMENTAL PROCEDURES**

Materials—Soybean (Glycine max, cv. Enrei), Arabidopsis thaliana (ecotype Columbia), Brassica napus, and black pine (Pinus thunbergii; a gift from Dr. K. Shinohara) were used.

Plasmids—The plasmid pHisADS, encoding unedited accD mRNA, was constructed by inserting the accD genomic DNA into the XhoI and BglII sites of pHisAD (18) instead of accD cDNA.

Expression and Purification—The methods used have been described previously (18) except that protease inhibitor tablets (Complete™, Roche Applied Science) were used.

The abbreviations used are: ACCase, acetyl-CoA carboxylase; CT, carboxyltransferase; PAGE, polyacrylamide gel electrophoresis.
EDTA-free, Roche Molecular Biochemicals) were added to the buffer for the purification step. Purification of recombinant His tag proteins followed the Novagen protocol. The column was washed with 5 column volumes of a washing buffer containing 60 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl (pH 7.9). A small portion of the recombinant protein from pHisADS leaked out during washing. The elute with a solution containing 150 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl (pH 7.9) was used for enzyme assay.

**Immunoprecipitation**—Immunoprecipitates were obtained by anti-β polypeptide IgG and protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) (18).

**Enzyme Assay**—CT activity and ACCase activity were measured as described (18).

**Sequence**—Total RNA and DNA were prepared from seedlings of soybean, Arabidopsis, Brassica, and black pine described by Kozaki et al. (18). cDNA synthesis, polymerase chain reaction, and direct DNA sequencing were carried out (18).

**Multiple Alignments**—The amino acid sequences of accD genes were aligned using the CLUSTAL W program (19). Because the sequence similarities and eukaryotic forms of ACCase are low, we performed the sequence similarity search using the UCSC SAM-T98 program with default parameters (20). This method uses an iterative hidden-Markov model and uncovers subtle relationships missed by single-pass data base search methods (21, 22). The amino acid sequences of E. coli accD gene were used as a query. The amino acid sequences of eukaryotic forms of ACCase, ACC1, are aligned with those genes based on the result of this search.

**RESULTS**

**Comparison of Unedited and Edited Enzymes**—Pea accD consists of 1,770 base pairs and encodes the β polypeptide of 590 amino acid residues (23). Only one editing site, where a cytosine is changed into a uracil, has been found at nucleotide position 800 (18). This corresponds to the second position (underlined) of the 267th codon, a UCG (serine) codon that is converted into a UUG codon (leucine) in the mRNA. The cDNAs encoding accA mRNA and either the unedited or edited accD mRNA were bicistronically inserted into the pET-19b vector, and we obtained two expression plasmids, pHisADS (Fig. 1A) and pHisAD. The difference between the two plasmids is a base at position 800.

The pHisADS plasmid was expressed in E. coli. The resultant CT complex was partially purified using a nickel column and compared with that resulting from the expression of pHisAD (Fig. 1, B and C). Upon SDS-PAGE, both fractions gave a major band with a molecular mass of 102 kDa and two minor bands of 72 and 87 kDa. The 102-kDa band reacted with the anti CT complex was partially purified using a nickel column. In addition, when anti-β polypeptide serum was added to the fraction (50 μg of protein) purified on a nickel column, the His tag was fused to the α polypeptide, and the β polypeptide copurified on a nickel column. In addition, when anti-β polypeptide IgG was added to the eluate from the nickel column, the α polypeptide coprecipitated with the β polypeptide (Fig. 1D). These results indicate that the β polypeptide is associated with the α polypeptide. The abundance of the α polypeptide exceeds that of the β polypeptide (Fig. 1B), indicating a difference in expression of the two polypeptides. A certain amount of the α polypeptide was not associated with the β polypeptide, as shown previously (18), and a small portion of the α polypeptide associated with the β polypeptide. There was almost no difference of antigenicity between the eluates from pHisAD and pHisADS. Thus, the level of expression of the unedited complex is similar to that of the edited complex.

Both the unedited and edited complexes were mainly eluted by a buffer containing 150 mM imidazole and 500 mM NaCl from a nickel column. Freezing and thawing of the eluate did not affect the solubility of the edited complex, but affected the unedited complex, resulting in an insoluble complex. Dialysis of the eluate of the unedited complex against a buffer containing 50 mM Tricine-KOH (pH 8) and 150 mM NaCl resulted in precipitates. In contrast to this, the edited complex was soluble, even in 50 mM Tricine-KOH (pH 8). Thus, the solubility of the unedited complex was different from that of the edited complex. A serine residue at position 267 is probably not compatible with the formation of a soluble complex with the α polypeptide, whereas a leucine residue is required.

The eluate from the nickel column obtained from the expression of pHisAD had CT activity as reported previously (18) and had ACCase activity when a complex of biotin carboxylase with the biotin carboxyl carrier protein was added (Table I). However, the fraction from pHisADs had neither CT nor ACCase activity upon addition of the biotin carboxylase complex, although the unedited complex was soluble and did not precipitate under the assay conditions used. To find any CT activity of the unedited complex under different conditions, we also assayed under an altered malonyl-CoA (0.1–1 mM) and salt concentration (0–100 mM NaCl) but did not detect any activity. The protein degradation in both eluates was not observed during incubation. These results indicate that the amino acid at position 267 is important and that RNA editing in accD mRNA.

**Figure 1. Expression of pHisAD and pHisADS.** A, the plasmid pHisADS, encoding unedited accD mRNA, was constructed by inserting the accD genomic DNA into the XhoI and BplI sites of pHisAD (18) instead of accD cDNA. Into the pet19b vector, accA cDNA, a ribosomal binding site (rbs), and accD genomic DNA were inserted. The asterisk indicates the only difference between pHisADS and pHisAD. The base in pHisADS at this position was cytosine and that in pHisAD was uracil. B, SDS-PAGE of the crude extract from E. coli (55-μl culture) containing pHisAD or pHisADS and the fraction (6 μg of protein) purified on a nickel column. The gel was stained with Coomassie Brilliant Blue. C, immunoblots. The fraction (0.6 μg of protein) purified on a nickel column was separated by SDS-PAGE and probed with an anti-α or anti-β polypeptide serum. D, immunoprecipitates. Anti-β polypeptide IgG was added to the fraction (50 μg of protein) purified on a nickel column, and the precipitates separated by SDS-PAGE were stained with Coomassie Brilliant Blue.
RT activity from a nickel column was measured as described previously (18). ACCase activity was measured after the addition of a biotin carboxylase complex with a biotin carboxyl carrier protein prepared by anion exchange chromatography of a pea chloroplast extract (18). This specific activity was expressed on the basis of the amount of the recombinant CT protein. Results are the means of two independent determinations. Almost the same results were obtained for two independent enzyme preparations.

| Fraction containing | Edited β polypeptide | Unedited β polypeptide |
|---------------------|----------------------|------------------------|
| CT activity         | 3020                 | 0                      |
| ACCase activity     | 980                  | 0                      |

**TABLE I**

Enzyme activities

CT activity of the eluate from a nickel column was measured as described previously (18). ACCase activity was measured after the addition of a biotin carboxylase complex with a biotin carboxyl carrier protein prepared by anion exchange chromatography of a pea chloroplast extract (18). This specific activity was expressed on the basis of the amount of the recombinant CT protein. Results are the means of two independent determinations. Almost the same results were obtained for two independent enzyme preparations.

*FIG. 2.* Similarities among the accD and ACC1 sequences around the RNA editing site. The arrow indicates the RNA editing site of pea accD. Amino acid residues identical in half or more of the sequences are shaded in black, and conservative substitutions are shaded in gray. The left column indicates species abbreviations as follows: Pea, Pisum sativum; Soybean, G. max; Arabidopsis, A. thaliana; Brassica, B. napus; Dodder, Cuscuta reflexa; Pine, P. thunbergii; Plastid 1, Picea abies; Plastid 2, Nicotiana tabacum; Plastid 3, Solanum tuberosum; Plastid 4, Spinacia oleracea; Plastid 5, Oenothera elata subsp. Hookeri; Plastid 6, Epipagus virginiana; Plastid 7, Angiopteris lygodiiifolia; Plastid 8, Marchantia polymorpha; Plastid 9, Physcomitrella patens; Plastid 10, Chlorella vulgaris; Plastid 11, Cynidium caldarium; Plastid 12, Nephrolepis ollivacea; Plastid 13, Porphyra purpurea; Cyan 1, Synechocystis PCC6803; Cyan 2, Synechococcus PCC7942; Bacterium 1, E. coli; Bacterium 2, Bacillus subtilis; Eukaryote 1, Saccharomices cerevisiae; Eukaryote 2, A. thaliana (cytosolic ACCase); Eukaryote 3, Homo sapiens.

is required for CT activity and therefore for ACCase activity in vitro. Probably the β polypeptide, having a serine residue instead of leucine at position 267, formed a complex of inappropriate conformation for catalytic activity.

Occurrence of RNA Editing in Other Plants—The in vitro evidence described above suggests that RNA editing is required for functional enzyme in vivo. To examine this proposition, we looked at whether RNA editing occurs in plants not having the leucine codon necessary for a functional enzyme at the position in question of the accD gene. If RNA editing occurs to create a leucine codon at the same position in such plants, we can conclude that editing is needed to form in vivo functional CT. For the Southern Asian dodder, soybean, Arabidopsis, and Brassica accD gene is UUG, as in peas, and the proline codon of the black pine accD gene is CCA. RNA editing in chloroplasts is mostly a cytosine-to-uracil change at the second nucleotide position of the triplet (14). If a cytosine of the second nucleotide of UCG (serine) and CCA (proline) is converted to a uracil, the resultant UUG and CUA triplets encode a leucine, and there is a possibility such editing to occur.

Next, to survey possible occurrence of editing, we compared each codon at the position 267. The data showed that the serine codon of the Southern Asian dodder, soybean, Arabidopsis, and Brassica accD gene is UCG, as in peas, and the proline codon of the black pine accD gene is CCA. RNA editing in chloroplasts is mostly a cytosine-to-uracil change at the second nucleotide position of the triplet (14). If a cytosine of the second nucleotide of UCG (serine) and CCA (proline) is converted to a uracil, the resultant UUG and CUA triplets encode a leucine, and there is a possibility such editing to occur.

To examine whether such editing does occur, we compared the nucleotide sequences of the accD gene and its cDNA from soybeans, Arabidopsis, Brassica, and black pine by direct DNA sequencing (Fig. 3). The observed gene sequence of the soybeans, Arabidopsis, and Brassica accD at the position in question were all TGG, which agreed with the reported data, but the cDNA sequences were all converted to TGG. The observed sequence of the black pine gene is CCA, which agreed with the reported data, but the sequence of its cDNA was converted to CTA. Thus, cytosine residue at the second position of the triplet was converted to a uracil at mRNA. These results indicate that the predicted editing occurred and that a leucine codon was
created by RNA editing. Thus, editing at the same position was also shown to take place in the β polypeptide mRNA in all the plants tested, indicating that editing is needed to synthesize a functional enzyme in vivo and is necessary for ACCase of these plants.

**DISCUSSION**

The experiments reported here are the first demonstrations that chloroplast RNA editing is required for a functional ACCase, using two different approaches. One is the biochemical demonstration that the unedited recombinant enzyme is inactive in contrast to the edited enzyme. This means that editing is essential for in vitro enzyme activity. The second is the demonstration that chloroplast RNA editing takes place to create the leucine codon in all the examined accD gene not having leucine or its similar amino acid codon necessary for a functional enzyme. This suggests the necessity of RNA editing for a functional enzyme in vivo. We propose here that in plants RNA editing to create the leucine codon is essential in vivo.

The most frequently edited chloroplast RNAs are probably the ndh transcripts encoding a putative chloroplast NADH dehydrogenase. For example, tobacco ndhA, ndhB, and ndhD transcripts have been reported to have two, nine, and two editing sites, respectively (11, 13). The extent of editing in the ndhD transcript depends on developmental and environmental conditions (24). These genes are shown to be dispensable for plant growth under mild environmental conditions (25, 26), and the biological significance of RNA editing in ndh transcripts is not yet understood.

A possible role of chloroplast RNA editing has been reported for the tobacco psbF transcript encoding the α subunit of the chloroplast-encoded RNA polymerase (13). This editing is believed to be involved in the regulation of RNA polymerase activity, because the extent of editing depends on developmental conditions. However, it is not known whether both the unedited and edited enzymes are functional. Further experiments are needed to characterize the biological significance.

In contrast to ndh transcripts, the biological significance has been demonstrated for psbF (27) encoding a core component of the photosystem II and for petB (28) encoding a subunit of the cytochrome b6f complex. psbF is required for the functional photosystem II (29). Spinach and tobacco psbF proteins are 100% identical, but only a nucleotide of the spinach transcript is edited. The introduction of spinach psbF into the tobacco plastid genome that lacks the capacity to edit the introduced site resulted in a mutant phenotype of slower growth, lowered chlorophyll content, and high chlorophyll fluorescence. This lack of editing resulted in reduced protein activity, but not a complete loss of function, indicating that this RNA editing is not essential for in vivo function. Chlamydomonas and maize petB proteins are 88% identical (94% similar). A codon at position 204 of the petB gene is leucine in Chlamydomonas, but in maize it is proline, which is changed to a leucine by RNA editing. To examine a possible role of proline at the position 204, a proline codon was introduced in place of a leucine codon editing. To examine a possible role of proline at the position 204 of the petB gene of Chlamydomonas. Chloroplast transcripts are not edited in Chlamydomonas. The Chlamydomonas transformants obtained were nonphototropic mutant. They lacked photosynthetic electron transfer and cytochrome b6f activity, indicating that the proline is not fit for Chlamydomonas cytochrome b6f. This result strongly suggests that this RNA editing is essential for photosynthesis in maize.

Plants have two forms of ACCase, the heteromeric, prokaryotic form composed of four subunits in chloroplasts, and the homomer, eukaryotic form composed of a single polypeptide in cytosol, except for Gramineae, which lacks the accD gene (30, 31). Although rice has an accD gene remnant, wheat and maize do not have a counterpart to this. Gramineae does not have the prokaryotic form of ACCase in chloroplasts but has the nuclear-encoded eukaryotic form of ACCase in both chloroplasts and cytosol. Each ACCase supplies malonyl-CoA for the synthesis of fatty acids in chloroplasts or for the synthesis of flavonoid and the chain elongation of fatty acids in cytosol. To our knowledge no evidence exists that malonyl-CoA synthesized in cytosol enters into plastids. ACCase is necessary in chloroplasts for de novo fatty acid synthesis. Probably the prokaryotic form of ACCase is essential for plants. E. coli accD is an essential gene (32). Both accA and accD are present as a single copy in Arabidopsis nuclear and chloroplast genomes, respectively, and are essential genes. Thus, chloroplast RNA editing is necessary not only for ACCase but also for the survival of these plants.

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