Isolation and Characterization of cDNA Clones for the E1β and E2 Subunits of the Branched-chain α-Ketoacid Dehydrogenase Complex in Arabidopsis*

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Branched-chain α-ketoacid dehydrogenase (BCKDH) has been known in mammals to be a key enzyme of the catabolic pathway of branched-chain amino acids. We have isolated two cDNA clones encoding the E1β and E2 subunits of BCKDH, respectively, from Arabidopsis thaliana. Proteins encoded in these cDNA sequences had putative mitochondrial targeting sequences and conserved domains reported for their mammalian counterparts. Northern blot and immunoblot analyses showed that transcripts from the respective genes and E2 protein markedly accumulated in leaves kept in the dark. We found that the activity of BCKDH in the leaf extracts also increased when plants were placed in the dark. Addition of sucrose to detached leaves inhibited the accumulation of transcripts, whereas application of a photosynthesis inhibitor strongly induced the expression of these genes even under light illumination. These observations indicate that the cellular sugar level is likely responsible for the dark-induced expression of these genes. The transcript levels of these genes were also high in senescing leaves, in which photosynthetic activity is low and free amino acids from degraded protein are likely to serve as an alternative energy source.

The mammalian branched-chain α-ketoacid dehydrogenase (BCKDH)1 is a mitochondrial multienzyme complex that is composed of three subunits carrying different enzymatic activities: branched-chain α-ketoacid decarboxylase (E1; EC 1.2.4.4), dihydrolipoyl transacylase (E2; no EC number), and dihydrolipoamide dehydrogenase (E3; EC 1.8.1.4). The E1 subunit is further composed of two E1α and two E1β subunits. This enzyme complex also contains two specific regulatory enzymes, a kinase and a phosphatase (1). E1 catalyzes the oxidative decarboxylation of branched-chain α-keto acids, which are derived from branched-chain amino acids by transamination. E2 catalyzes the transfer of the acyl group from the lipoic moiety to coenzyme A. E3 is a flavoprotein and reoxidizes the reduced lipoil sulfur residues of the E2 subunit (2).

BCKDH in mammals is thought to be a key enzyme in the catabolism of the branched-chain amino acids, i.e. valine, leucine, and isoleucine. As end products, leucine is converted to acetyl-CoA and acetoacetate, valine to succinyl-CoA, and iso-leucine to succinyl-CoA and acetyl-CoA (3). Through this pathway, these amino acids serve as substrates for energy production via acetoacetate and succinyl-CoA. It has been firmly established that nutritional conditions play an important role in modulating the activity of BCKDH through a phosphorylation-dephosphorylation mechanism (4). From this point of view, BCKDH is critically important in the pathway for energy utilization, rather than being merely a system for the catabolism of a small group of amino acids.

BCKDH has been extensively studied in mammals because defects in the genes for this enzyme cause maple syrup urine disease, an inborn disorder of metabolism in humans (3). In the plant kingdom, however, we have come across only one case reporting the detection of the activity of this enzyme in mung bean (Vigna radiata L.) (5, 6).

Plants employ complicated mechanisms to adapt themselves to various environmental conditions. As the energy source for photosynthesis, light is a vital environmental factor for the plant. Unfavorable light conditions such as prolonged darkness and shading lead to a significant decrease in the photosynthetic activity of leaves, and the carbohydrate reserves are quickly depleted (7, 8). The metabolic consequences of carbohydrate starvation have been studied in a number of plant species (9–13). After a long duration of starvation, many cellular constituents are degraded and used as an energy source for survival. This process has not been fully interpreted, however, in terms of molecular events. We have identified genes that are strongly expressed in leaves when they are placed in the dark. As an initial part of our efforts, the din1 gene was isolated from cotyledons of dark-treated radish (Raphanus sativus L.) (14, 15). So far, we have isolated more than one dozen cDNA clones for genes that are strongly expressed in Arabidopsis thaliana leaves in the dark, using differential display reverse transcription-polymerase chain reaction. Two of these cDNA clones had sequence similarities to the genes for the E1β and E2 subunits of BCKDH, respectively (16). Isolation of these cDNAs has not only provided the first structural information on these proteins in plants, but has also given information on the expression of the corresponding genes that would suggest the physiological role of the enzyme during carbohydrate starvation.

EXPERIMENTAL PROCEDURES

Plant Materials—Plants (Arabidopsis L. ecotype Columbia) were grown at 23 °C under continuous light illumination (60 μmol/m2/s) and supplied with Arabidopsis inorganic medium (17). For dark treatment, young plants grown for 3 weeks were exposed to complete darkness for various time periods. Sugars and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were supplied to detached leaves as described by Mita et al.

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1 The abbreviations used are: BCKDH, branched-chain α-ketoacid dehydrogenase; E1, branched-chain α-ketoacid decarboxylase; E2, dihydrolipoyl transacylase; E3, dihydrolipoamide dehydrogenase; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; bp, base pair.
(18). The leaves were incubated in the dark for 48 h with 3% sucrose or mannitol. The leaves were also floated on water containing 10 μM DCMU with or without 3% sucrose for 48 h under continuous light illumination. For sampling of plant organs and naturally senescing leaves, plants were grown under a 16-h light and 8-h dark photoperiod. Samples were harvested from plants of 1 month old. Leaves from ~60-day-old plants were divided into four groups according to the extent of yellowing of leaves. Stage M represents fully expanded mature leaves and stages S1, S2, and S3 represent leaves with ~25, 50 and 75% of the leaf area visually estimated to have yellowed, respectively.

Differential Display Reverse Transcription-Polymerase Chain Reaction—Plants grown for 3 weeks under continuous light illumination were exposed to darkness or kept under light illumination for 24 h. Total RNA was extracted from the leaves according to the method of Schmidt et al. (19). poly(A) + RNA was separated from total RNA using oligo(dT)-cellulose (Takara, Kyoto, Japan). First-strand cDNA was synthesized by reverse transcription of the poly(A) + RNA (Amersham Pharmacia Biotech). Differential display reverse transcription-polymerase chain reaction analysis was performed as described by Yoshida et al. (20). The polymerase chain reaction was performed with 2 ng of the first-strand cDNA and 10 μM 10-mer primer (Operon Technologies, Inc., Alameda, CA). After 40 amplification cycles at 94 °C for 1 min, 35 °C for 1 min, and 72 °C for 2 min, followed by 72 °C for 10 min, the amplified cDNAs were separated on a 1.5% agarose gel and the positive fragments were recovered from the gel and subcloned into the pCR II vector (Invitrogen, San Diego, CA). The expression of the genes corresponding to these fragments was examined by Northern hybridization with the respective fragments as probes.

Construction and Screening of a cDNA Library—poly(A) + RNA from leaves exposed to darkness for 24 h was used as a template for cDNA synthesis. Double-stranded cDNA was synthesized using a TimeSaver cDNA synthesis kit (Amersham Pharmacia Biotech) as recommended by the manufacturer. The cDNA products were inserted into the λ-ZAPII vector (Stratagene). Phage plaques were blotted on nylon membranes (Biodine A, Pall Bio Support, East Hills, NY) by the standard plaque lift method (21). 32P-Labeled cDNA probes were prepared by a Megaprime labeling system (Amersham Pharmacia Biotech). Hybridization was carried out at 65 °C overnight according to Sambrook et al. (21). The positive clones were converted to plasmid clones by in vivo excision according to the manufacturer’s instruction (Stratagene).

DNA Sequencing—Nucleotide sequences were determined by the dideoxynucleotide chain termination method (22) using an automated DNA sequencer (Pharmacia LKB Biotech). The expressed protein accumulated in Escherichia coli strain BL21(DE3). Hybridization was performed at 65 °C overnight according to the manufacturer’s protocol (Promega). The expressed protein accumulated in inclusion bodies, was purified as described by Harlow and Lane (24), and was then separated by SDS-polyacrylamide gel electrophoresis (25). After the gel was stained with Coomassie Blue R-250, the major band with the expected mobility was excised from the gel and used for the preparation of an antibody against the purified protein in rabbits.

Immunoblot Analysis—Leaf extracts were prepared by grinding the frozen tissue in an extraction buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM NaCl, 5 mM ascorbic acid, 0.05% β-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride. Protein concentration was determined by the method of Bradford (26) with a protein assay kit (Bio-Rad). Soluble proteins were denatured in an equal volume of 2× SDS buffer and separated by SDS-polyacrylamide gel electrophoresis (25). The separated polypeptides were electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore Corp.). The membrane was blocked with blocking solution (5% nonfat dry milk and 0.25% Tween 20 in Tris-buffered saline) for 2 h. The membrane was then incubated with the primary antibodies against the E2 protein for 1 h. After washing with blocking solution, the blots were incubated for 1 h with alkaline phosphatase-conjugated goat anti-rabbit IgG (Organon Teknika, West Chester, PA), which was diluted 1:4000. The color reaction was performed by incubation of the membrane in 100 mM Tris-HCl, pH 9.6, 100 mM NaCl, 4 mM MgCl2, 0.1 mM nitro blue tetrazolium, 0.05 mM 5-bromo-4-chloro-3-indolyl phosphate, and 1% dimethylformamide.

Extraction and Assay of BCKDH Activity—Extraction and assay of BCKDH were performed according to Goodwin et al. (27) with minor modifications as described below. Leaves were disrupted with a Polytron homogenizer in an extraction buffer containing 50 mM HEPES, pH 7.5, 20% (v/v) glycerol, 200 mM KCl, 3 mM EDTA, 0.5% (v/v) Triton X-100, 1 mM dithiothreitol, 0.1 mg/ml leupeptin, 0.2 mg/ml pepstatin A, and 0.1 mg/ml phenylmethylsulfonyl fluoride. After centrifugation for 60 min at 100,000 × g, the supernatant was saved, and its volume was measured. 0.25 volume of 45% (w/v) polyethylene glycol 8000 was added to the supernatant to give a final polyethylene glycol concentration of 9%. After stirring on ice for 30 min, the extract was centrifuged at 15,000 × g for 10 min. Bands corresponding to the extraction buffer with a Potter-Elvehjem-type homogenizer were removed by centrifugation at 100,000 × g for 1 h. The pellets were resuspended in the extraction buffer with a Potter-Elvehjem-type homogenizer and desalted with PD-10 columns (Amersham Pharmacia Biotech).

The activity of BCKDH was determined spectrophotometrically by measuring the reduction of NAD+ at 340 nm. The assay mixture contained 175 mM Tris-HCl, pH 7.5, 7.5 mM MgCl2, 0.3 mM NAD+, 0.6 mM CoASH, 0.4 mM thiamin pyrophosphate, 0.1% (v/v) Triton X-100, 1 mM NaCN, and the extract (0.4–0.6 mg of protein in 600 μl of reaction solution). The reaction was initiated with 0.1 mM α-keto-β-methylvalerate, and the blank rate determined in the absence of substrate was subtracted. The assay was performed at 23°C, and 1 unit of enzymatic activity is expressed as micromoles of NADH formed per min/g of tissue (fresh weight).

RESULTS

Isolation of cDNA Clones for the E1β and E2 Subunits of BCKDH—In an attempt to isolate cDNAs corresponding to genes that are activated in leaf cells in the dark, we were able to identify dozens of cDNA fragments by a simplified differential display procedure. Dark-specific expression of the genes was confirmed by Northern hybridization with the respective cDNA fragments as probes (data not shown). We found that two of these cDNA fragments, din3 and din4, encoded proteins with striking amino acid sequence similarities to the E2 and E1β subunits of mammalian BCKDH, respectively. As there has been no information on plant BCKDH genes, we isolated full-length cDNA clones from a cDNA library prepared from Arabidopsis leaves that were placed in the dark for 24 h.

The din3 cDNA clone contains a 1449-base pair (bp) open reading frame encoding a protein of 483 amino acid residues (Fig. 1) with a predicted molecular mass of 55 kDa. Nucleotide sequence analysis demonstrated that this cDNA encoded a protein with significant homology and 95% identity to human (28) and bovine (29) E2 subunits (Fig. 1). The mammalian E2 subunit is known to contain three functional domains: a lipoate-containing domain, an E1- and E3-binding domain, and a catalytic domain (1, 30). The lipoate-containing domain is located between residues 105 and 117, and the E1- and E3-binding domain is located between residues 175 and 206 of the human E2 protein (31). The catalytic domain is located in the C-terminal portion of the protein (29, 30). There is a very strong homology between the lipoate-binding domain from mammalian proteins and the E2 protein from Arabidopsis (amino acids 111–127), with 71% identity and 88% similarity compared with human E2 (Fig. 1). A lipoyllysine residue in the lipoate-containing domain is highly conserved among mammalian E2 proteins. There is also a lysine residue in the Arabidopsis E2 protein at an identical position (Lys-116) correspond-
ing to the lipoyllysine residues in the mammalian proteins (Fig. 1). The E1- and E3-binding domain is also conserved, with 56% identity and 78% similarity between the human and Arabidopsis (residues 185–216) E2 proteins. The C-terminal catalytic domains of mammalian E2 proteins contain a His residue in a conserved sequence, His-Xaa-Xaa-Xaa-Asp-Gly (32, 33). The putative catalytic domain of the Arabidopsis protein contains a His residue prior to the C terminus (Fig. 1). It has been postulated that His in this position is involved in CoASH bonding for catalyzing the covalent linkage of the thiol ester to the acyl group (32). The fact that these domains, including the lipoyllysine residue and CoASH-bonding motif, are conserved among species suggests that they have critical functions in the plant protein as well.

The cDNA clone for the din4 gene contains a 1074-bp open reading frame encoding a protein of 358 amino acid residues with a predicted molecular mass of 39 kDa. This amino acid sequence is highly similar to that of the mammalian BCKDH E1b subunit (Fig. 2). The amino acid sequence encoded by din4 cDNA showed 59 and 58% identities to the human (34) and bovine (35) amino acid sequences, respectively.

Southern Blot Analysis of Genomic DNA—We performed Southern blot analysis to estimate the copy number of the genes for the E1b and E2 subunits from Arabidopsis. Genomic DNA was digested with BamHI, HindIII, EcoRI, PstI, or EcoRV. There is one HindIII site in the din4 cDNA, but no other restriction sites in both cDNAs. The din3 cDNA hybridized to only one restriction fragment (Fig. 3). In contrast, the din4 cDNA hybridized to three bands in the HindIII digests and to two bands in the other restriction digests (Fig. 3). Furthermore, we performed Southern blot analysis to examine whether the appearance of three bands in the HindIII digests was due to the presence of a HindIII site in the din4 cDNA. The din4 cDNA was cut into two fragments at the HindIII site, and each fragment was used as a probe for Southern blot analysis. One fragment hybridized to 700- and 1000-bp bands, and the other hybridized to 1000- and 2500-bp bands (data not shown). Hence, the 700- and 2500-bp bands corresponded to a gene complementary to the din4 cDNA, and the 1000-bp band corresponded to a homologous gene. Luethy et al. (36) have isolated another cDNA clone for the E1b gene in Arabidopsis. Given this, the Arabidopsis genome seems to contain two copies of the E1b gene and a single copy of the E2 gene.

Expression of the Genes for the E1b and E2 Subunits during Dark Treatment—We examined the kinetics of the dark-induced expression of the genes for the E1b and E2 subunits by Northern blot analysis. The expression kinetics of the genes for the E1b and E2 subunits were very similar to each other (Fig. 4, A and B). The transcripts from both genes were barely detectable in leaves grown under continuous light conditions, but they could be detected 3 h after the beginning of the dark treatment and continued to accumulate. At 48 h in the dark,
the levels of the transcripts from both of the genes reached a maximum, and then the transcript levels decreased gradually.

To examine the accumulation level of the E2 protein in leaves, we used antibodies against the E2 subunit. Fig. 4C shows that polypeptides with an apparent molecular mass of 52 kDa in leaf extracts were specifically recognized by the antibodies. As shown in Fig. 4C, dark treatment caused the increase in the content of E2 protein. The accumulation of the E2 protein appeared to parallel that of the mRNA.

Assay of BCKDH Activity—We measured the activity of the active form of BCKDH in leaves that were placed either in the dark or under light illumination for 3 days. The procedure was based on studies in mammalian tissues (27, 37), but there were several modifications as described below. Since mammalian BCKDH has been known to be freed of endogenous E3 during polyethylene glycol precipitation, E3 has been added to the assay solution (27). Under our experimental conditions, however, the reduction of NAD was not stimulated by 10 units of porcine E3. The possibility of interconversion during homogenization was checked by investigating the effect of inhibitors of phosphatase (100 mM NaF) and kinase (5 mM α-ketoisocaproate or 1 mM dichloroacetate). Application of these inhibitors with the extraction buffer had little effect on the final activity of the enzyme.

The activity of BCKDH from dark-treated leaves was 0.89 ± 0.13 milliunits/g of tissue (mean ± S.D., n = 3), which was

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**Fig. 2. Alignment of the deduced amino acid sequence of the Arabidopsis E1β protein with those of the human and bovine E1β subunits.** Black boxes indicate identical amino acid residues.

**Fig. 3. Southern hybridization of Arabidopsis genomic DNA.** Genomic DNA (1 µg) was digested with BamHI (B), HindIII (H), EcoRI (E), PstI (P), or EcoRV (V). Left panel, E2; right panel, E1β. Molecular size markers (in base pairs) are indicated on the left.

**Fig. 4. Time course analysis of the gene expression of the E1β and E2 subunits during dark treatment.** A, total RNA (10 µg) from leaves harvested at the indicated times during dark treatment was used for Northern blot analysis. Total RNA was stained with ethidium bromide (lower panel). B, the relative levels of transcripts from the E1β (open circles) and E2 (closed circles) subunit genes were determined by quantitation of hybridization signals by BAS2000 imaging analyzer. The results are expressed as a percentage of the maximum level. C, soluble protein (5 µg) from leaves harvested at the indicated times during dark treatment was used for immunoblot analysis with antibodies against E2 subunits.
the organ-specific expression of the genes for the E1b and E2 subunits. Detached leaves were incubated with different media for 48 h either under continuous light illumination (lanes 1, 5, and 6) or in darkness (lanes 2–4). Lanes 1 and 2, water with no addition of sugar; lane 3, 3% sucrose; lane 4, 3% mannitol; lane 5, 10 μM DCMU; lane 6, 10 μM DCMU plus 3% sucrose. Total RNA (20 μg) from each sample was analyzed by Northern blot hybridization.

The activity of light-grown plants was below the detection limit of the assay. When 50 μl of leaf extracts were preincubated with 5 μl of antibodies to E2 for 20 min on ice prior to the assay, the production of NADH was completely inhibited, whereas preimmune serum had no effect (data not shown). These data suggest that the enzymatic activity we have detected involves the catalytic function of the E2 protein, which is encoded by the din3 gene, and that the activity increases in leaves kept in the dark, which parallels the accumulation of its transcripts and proteins.

Metabolic Control of E1β and E2 Subunit Expression by Sugars—We searched the regulatory signals that are responsible for the dark-induced gene expression of the E1β and E2 subunits. One of the proposed physiological effects of dark treatment is the cessation of photosynthesis in leaves, resulting in the lowering of the sugar level in leaf cells. To address the question of gene activation as a consequence of sugar deprivation, we examined the effect of exogenously supplied sugars and the photosynthesis inhibitor DCMU (Fig. 5). Transcripts from the genes for the E1β and E2 subunits accumulated in detached leaves floated on a 10 μM DCMU solution for 2 days, even under light illumination. In contrast, when leaves were floated on a 3% sucrose solution for 2 days, accumulation of the transcripts was totally suppressed both in darkness and under light illumination in the presence of DCMU. These results suggest that the expression of the genes for the E1β and E2 subunits is modulated by the sugar level in leaf cells.

Organ-specific Expression of Plant BCKDH—We examined the organ-specific expression of the genes for the E1β and E2 subunits in plants by Northern blot analysis (Fig. 6). The transcripts from both genes were less abundant in leaves, cauline leaves, and bolts (stems), which possess photosynthetic activity. The transcripts were more abundant in non-photosynthetic organs such as siliques, flowers, and roots, in which the level of free sugars is considered to be low.

Expression of the Genes for BCKDH during Leaf Senescence—In senescing leaves as well as in sugar-starved cells, proteins are massively degraded, and amino acids are used as an energy source. We observed that a gradual loss of cellular protein occurred in senescing leaves (data not shown). We examined the expression of the genes for E1β and E2 in senescing rosette leaves by Northern blot analysis. For simple classification of leaves in terms of the extent of senescence, we visually inspected the yellowing of rosette leaves and divided them into four stages (see “Experimental Procedures”). The transcripts from genes for E1β and E2 were barely detectable in non-senescent green leaves (stage M) (Fig. 7). The transcripts from both genes accumulated slightly at the early stages of leaf senescence (S1 and S2), and the relative levels of transcripts were maximal at the last stage (S3), being ~20 times higher than in non-senescent green leaves (stage M). These results suggest that the expression of the E1β and E2 genes is strongly associated with the progression of leaf senescence.

DISCUSSION

This report presents the first evidence of the structural similarity of BCKDH from plants to the mammalian counterpart. We further propose the physiological role of BCKDH in plants from the results of examination of the gene expression of the E1β and E2 subunits of BCKDH in Arabidopsis.

BCKDH belongs to a 2-oxoacid dehydrogenase multienzyme complex family, namely pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (1). The amino acid sequence of the BCKDH E1β subunit has low but significant homology to that of the pyruvate dehydrogenase E1β subunit in mammals (35); the same is true for the BCKDH and pyruvate dehydrogenase E2 subunits (29, 38). The situation in plants is the same since the deduced amino acid sequences of the E1β and E2 subunits of Arabidopsis BCKDH share partial homology with those of the E1β and E2 subunits of Arabidopsis mitochondrial pyruvate dehydrogenase, respectively (39, 40). The amino acids sequence of the E2 subunit of BCKDH contained one highly conserved region that was identified as a lipoyl-binding site (Fig. 1). In contrast, the E2 subunit of pyruvate dehydrogenase from Arabidopsis has two lipoyl-binding sites (40), as is the case with the mammalian counterpart.

Mammalian BCKDH is known to be a mitochondrial enzyme (1). In higher plants, the oxidative decarboxylation of branched-chain α-keto acids has been shown to take place in peroxisomes (5, 6). It was suggested that further steps could also involve extraperoxisomal enzymes. Interestingly, plant β-methylcrotonyl-CoA carboxylase, which is involved in the degradation pathway of leucine, has been purified from mitochondria (41, 42). These studies predicted the presence of other key enzymes in the catabolism of branched-chain amino acids, including BCKDH, in plant mitochondria. In the present study, the E1β and E2 subunits of Arabidopsis BCKDH have been tentatively identified as mitochondrial proteins because of the presence of putative mitochondrial targeting sequences at the
suppressed the expression of the genes for the E1β and E2 subunits in rosette leaves. Stages of senescence were evaluated by the extent of yellowing of leaves. Stage M corresponds to mature green leaves. Stages S1, S2, and S3 correspond to the leaves in which ~25, 50, and 75% of the leaf area turned yellow, respectively. Total RNA (10 μg) extracted from leaves at each stage was analyzed by Northern blot hybridization. The Northern blot analysis showed that dark treatment induced the expression of the E1β and E2 genes in leaves. The accumulation of the transcripts began rapidly after leaves were exposed to darkness. In addition, the increase in the amount of the E2 protein and in the basal activity of BCKDH in the leaf extracts paralleled that of the accumulation of the corresponding mRNA during dark treatment. These results suggest that Arabidopsis BCKDH may play a role in the utilization of branched-chain amino acids as an alternative energy source in leaves in the dark when photosynthesis is hindered.

Light affects gene expression by altering the metabolic pools of carbohydrate produced by photosynthesis. Such a metabolic control may even override the direct effects of light and other forms of regulation (44, 45). Dark treatment blocks photosynthesis and causes carbohydrate starvation in leaf cells (46). A set of data indicated that carbohydrate starvation affects enzymatic activities and metabolism related to utilization of lipids and amino acids as an alternative carbon source (10, 47–49). Taken together with these observations, sugar starvation is very likely to be responsible for the expression of genes for BCKDH in plants. In addition, exogenously supplied sucrose suppressed the expression of the genes for the E1β and E2 subunits, and application of DCMU induced their expression under light conditions. Either dark treatment or the application of the photosynthesis inhibitor is likely to reduce the sugar level in leaf cells. These findings suggest that the cellular sugar level may serve as a physiological signal to regulate the expression of BCKDH genes.

The protein content of leaves declines progressively during sugar starvation (12). The protein degradation in starved cells causes an increase in the content of free amino acids, including branched-chain amino acids (50, 51). The catabolism of these amino acids generally involves compounds such as acetooacetate and succinyl-CoA, which enter the Krebs cycle (3). Aubert et al. (13) found that leucine, after asparagine, was the second most abundant amino acid that transiently accumulated in sycamore (Acer pseudoplatanus L.) cultured cells during carbohydrate starvation. The end product derived from leucine was thought to be used in place of sugars to fuel respiration in the mitochondria (13). Valine and isoleucine were also massively accumulated in sugar-starved sycamore cells (13). These studies support our suggestion that Arabidopsis BCKDH is involved in the utilization of branched-chain amino acids as an energy source in leaf cells when photosynthesis is hindered. Alternatively, BCKDH may be critically important in the pathway of nitrogen metabolism as well as energy utilization (52). Branched-chain amino acids are essential for protein synthesis (53), but excessive accumulation of branched-chain α-keto acids is toxic to animals (3). BCKDH seems to contribute to the disposal of these amino acids as evidenced by maple syrup urine disease (3). Although the toxicity of accumulation of excess branched-chain α-keto acids to plants has not been studied, plant BCKDH may also act to adjust the cellular balance of branched-chain amino acids and corresponding α-keto acids.

Northern analysis showed that the genes for the E1β and E2 subunits were strongly expressed in sink organs such as roots, flowers, and siliques in Arabidopsis. In these non-photosynthetic organs, carbohydrate consumption can be compensated only by utilization of exogenously supplied carbohydrates. However, it has been shown that symplastic diffusion of sugars from the veins toward the primary root tips cannot satisfy the carbon demand of the growing root meristem of maize (54). The situation may be similar in Arabidopsis (55). In contrast, it has been reported that plant tissues such as pea roots possess the capacity to use branched-chain amino acids as a respiratory substrate (56). Our data suggest that BCKDH plays a role in supplying sink organs with energy to supplement the energy demand of the cells.

We have shown that the accumulation of the transcripts from the E1β and E2 subunits was associated with the progress of senescence, suggesting that some molecular events are common to both dark-induced senescence and natural senescence. In Arabidopsis, the expression of several senescence-associated genes has been suggested to be directly coupled to the decline of photosynthetic activity, which reduces the cellular sugar levels in leaves (57). Since the transcripts from the E1β and E2 subunits accumulated in the senescing leaves, BCKDH may be involved in the utilization of the carbon skeletons of branched-chain amino acids as an energy source during leaf senescence as well as sugar starvation. The present study revealed that the genes of BCKDH represent a new class of molecular markers for the progression of leaf senescence in Arabidopsis. Further investigation with BCKDH will provide insight into the dynamic changes in metabolism occurring in the senescing leaves as well as in starved cells.

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REFERENCES
1. Yeaman, S. J. (1989) Biochem. J. 257, 625–632
2. Reed, L. J., Petit, F. M., Yeaman, S. J., Teague, W. M., and Biele, D. M. (1980) in Enzyme Regulation and Mechanism of Action (Mildner, P., and Ries, B., eds) Vol. 60, pp. 47–56, Pergamon Press, New York
3. Chuang, D. T., and Shih, V. E. (1995) in The Metabolic and Molecular Bases of Inherited Disease (Scrimer, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) 7th Ed., pp. 1239–1277, McGraw-Hill, Inc., New York
4. Reed, L. J., Damuni, Z., and Merryfield, M. L. (1985) Curr. Top. Cell. Regul. 27, 41–48
5. Gerbling, H., and Gerhardt, B. (1989) Plant Physiol. 91, 1387–1392
6. Gerbling, H. (1993) Bot. Acta 106, 380–387
7. Kerr, P. S., Ruffy Jr., T. W., and Huber, S. C. (1985) Plant Physiol. 78, 576–581
8. Stitt, M., Wirtz, W., Gerhardt, R., Heldt, H. W., Spencer, C., Walker, D., and Foyer, C. (1985) Planta (Heidelberg) 166, 354–364
9. Sagar, P. H., and Pratet, A. (1980) Plant Physiol. 66, 516–519
10. Journet, E. P., Bigny, R., and Douce, R. (1986) J. Biol. Chem. 261, 3193–3199
11. Brouquisse, R., James, F., Raymond, P., and Pratet, A. (1991) Plant Physiol. 95, 619–626
12. James, F., Brouquisse, R., Pratet, A., and Raymond, P. (1993) Plant Physiol. Biochem. 31, 845–856
13. Aubert, S., Albam, C., Bigny, R., and Douce, R. (1990) FEBS Lett. 283, 175–180
14. Azumi, Y., and Watanabe, A. (1991) Plant Physiol. 95, 577–583
15. Shimada, Y., Wu, G., and Watanabe, A. (1996) Plant Cell Physiol. 39, 139–143
16. Fujiy, Y., Sato, T., Yoshikawa, Y., Ito, M., and Watanabe, A. (1997) Plant Physiol. 114, (suppl.) S-95
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