Leucine and its keto acid enhance the coordinated expression of genes for branched-chain amino acid catabolism in *Arabidopsis* under sugar starvation

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Abstract Branched-chain α-keto acid dehydrogenase (BCKDH), a multienzyme complex, plays a key role in branched-chain amino acid catabolism. However, it remains unclear whether expression of each subunit is coordinately regulated in plants, which should be important for the efficient assembly of subunits into a functional multienzyme complex. We show that the transcripts from the *Arabidopsis* E1α subunit gene accumulated in dark-adapted leaves and in sugar-starved suspension cells. These results are complementary to our previous report that the transcripts for the E1β and E2 subunit genes accumulated in sugar-starved cells. Expression of the E1α gene is likely to be regulated by hexokinase-mediated sugar signaling, indicating that sugar plays a regulatory role in the coordinated expression of BCKDH subunit genes. Furthermore, Leu and its metabolite α-ketoisocaproate have synergistic effects on the enhanced expression of BCKDH subunit genes under sugar starvation. We hence suggest that branched-chain amino acids activate their own degradation pathway in sugar-starved cells through co-induction of each subunit gene of BCKDH. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Branched-chain amino acid; Branched-chain α-keto acid; Hexokinase; Sugar starvation; *Arabidopsis*

1. Introduction

Branched-chain α-keto acid dehydrogenase (BCKDH), a mitochondrial multienzyme complex consisting of E1α, E1β, E2, and E3 subunits, catalyzes oxidative decarboxylation of branched-chain α-keto acids produced by deamination of branched-chain amino acids, such as Leu, Ile, and Val [1,2]. Deficiency in any of these subunits results in the loss of BCKDH activity and causes accumulation of branched-chain amino acids and branched-chain α-keto acids, which are toxic to mammals [3]. BCKDH may be involved in scavenging excess amounts of branched-chain α-keto acids, because branched-chain α-keto acids have been found to induce apoptosis in yeast and mammalian cells [4,5]. BCKDH also plays a key role in energy production in mammalian brain and muscle via branched-chain amino acid catabolism [6,7]. BCKDH activity is strictly regulated by its substrates, branched-chain α-keto acids, which inhibit a protein kinase that specifically inactivates BCKDH [1–3,7]. Carbohydrate (glycogen) also affects branched-chain amino acid metabolism [8,9], whereas regulation of BCKDH gene expression by sugars has been reported in a few cases [10].

In plants, the presence of the BCKDH gene had not been demonstrated until we recently isolated cDNAs for the E1β and E2 subunits in *Arabidopsis* [11]. The transcripts from the E1β and E2 subunit genes accumulated in *Arabidopsis* leaves under sugar-limiting conditions such as darkness and senescence [11]. We also showed that BCKDH activity increased in dark-adapted leaves, suggesting that BCKDH plays a role in utilization of branched-chain amino acids as energy sources in place of sugars [11]. However, to understand fully the regulation of the gene expression of BCKDH, it is inevitable to investigate whether the expression of the E1α subunit is coordinately regulated with other subunit genes. Therefore, we herein examine the transcript levels of the *Arabidopsis* E1α subunit gene under sugar starvation. The results show that the kinetics of accumulation of the E1α transcripts in dark-adapted leaves and in suspension cells starved for sugars are similar to those of the E1β and E2 genes, which suggests that expression of BCKDH subunit genes is coordinately regulated under sugar starvation in plants. We further found that Leu and its catabolite α-ketoisocaproate (KIC) enhanced the expression of the BCKDH subunit genes in sugar-starved cells but not in sugar-fed cells. These results suggest that sugar plays a major role in the regulation of BCKDH gene expression, but Leu and KIC exert synergistic effects on the branched-chain amino acid catabolism under sugar starvation.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana L. (ecotype Columbia) plants were grown for 3 weeks under continuous illumination. Dark adaptation treatment and feeding of sucrose to leaves were carried out as described previously [12].

The *Arabidopsis* suspension-cultured cell line T87 [13] was main-

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Abbreviations: BCKDH, branched-chain α-keto acid dehydrogenase; Cab, chlorophyll *a/b* binding protein; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; KIC, α-ketoisocaproate; NR, nitrate reductase; MCCase, β-methylcrotonyl-coenzyme A carboxylase

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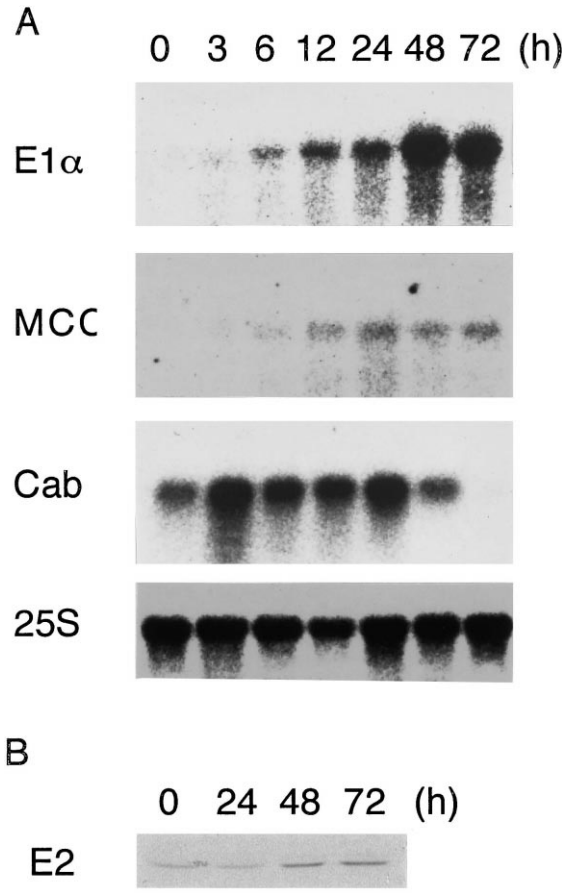

Fig. 1. Accumulation of the transcripts from the E1α subunit gene in dark-adapted *Arabidopsis* leaves. RNA and soluble protein extracts were isolated from leaves harvested at indicated times after the onset of dark adaptation. A: An equal amount of RNA (10 μg) was loaded in each lane. The membrane was probed with ^{32}P-labeled cDNA fragments for the E1α gene. As control, we examined the expression of the biotinylated subunit gene of MCCase (MCC) as a Leu catabolic enzyme and that of the *Cab* gene as a typical photosynthetic gene. The membranes were probed with 25S rDNA (25S), which is constitutively expressed during dark adaptation, to confirm the loading of equal amounts of RNA in each lane. B: Soluble cell extracts (5 μg protein) were used for immunoblot analysis with antibodies against the E2 protein.

tained in a culture medium described previously [14]. For the sugar starvation treatment, cells in exponential growth were collected from the culture medium, rinsed by successive resuspensions in a fresh medium devoid of sucrose, and then incubated in the sucrose-free medium. Leu or KIC (Sigma, St. Louis, MO, USA) was applied to the medium at a final concentration of 2 mM, and the pH of the medium was adjusted to 5.7.

2.2. RNA analysis

Isolation and gel blot analysis of RNA were performed as described previously [11]. The distribution of radioactivity on the membrane was analyzed with an Imaging Analyzer (BAS2000, Fuji Photofilm, Tokyo, Japan). cDNA clones for chlorophyll *a/b* binding protein (*Cab*, GenBank accession number W43139), nitrate reductase (NR2, AA042417) and the biotinylated subunit of β-methylcrotonyl-coenzyme A carboxylase (MCCase, H36836) were obtained from the Arabidopsis Biological Resource Center at Ohio State University. A cDNA fragment for the E1α subunit of BCKDH (AF077955, nucleotides +1224 to +1557) was amplified from an *Arabidopsis* cDNA by PCR. The membranes were reprobed with 25S rDNA as a control for relative loading of RNA in each lane. Each experiment was repeated twice, and similar results were observed in each case.

2.3. Protein analysis

Isolation and gel blot analysis of soluble protein extracts with antibodies raised against E2 protein were performed as described previously [11].

3. Results

3.1. Expression of the E1α gene under prolonged darkness

The transcript levels of the E1α subunit of BCKDH in the dark-adapted *Arabidopsis* leaves were monitored by RNA gel blot analysis. During dark adaptation, the transcripts from a photosynthetic gene, *Cab*, gradually declined (Fig. 1A). On the other hand, the E1α transcripts accumulated in dark-adapted leaves (Fig. 1A), with similar kinetics to those of the E1β and E2 transcripts [11]. As shown in our previous report [11], immunoblot analysis with antibodies raised against *Arabidopsis* E2 protein showed that a marked accumulation of the E2 polypeptide occurred in the dark (Fig. 1B). This suggested that the increase in the transcript levels of the BCKDH subunit genes was accompanied by accumulation of BCKDH proteins. As a positive control, we examined the transcript levels of the biotinylated subunit of MCCase, a well-known mitochondrial enzyme involved in the catabolic pathway of Leu [15,16]. The MCCase transcripts also accumulated in dark-adapted leaves (Fig. 1). All these results were complementary to our previous results on the E1β and E2 subunit genes, and therefore imply that each subunit gene of BCKDH and possibly other genes involved in branched-chain amino acid catabolism pathways are coordinately induced by sugar starvation occurring in darkness.

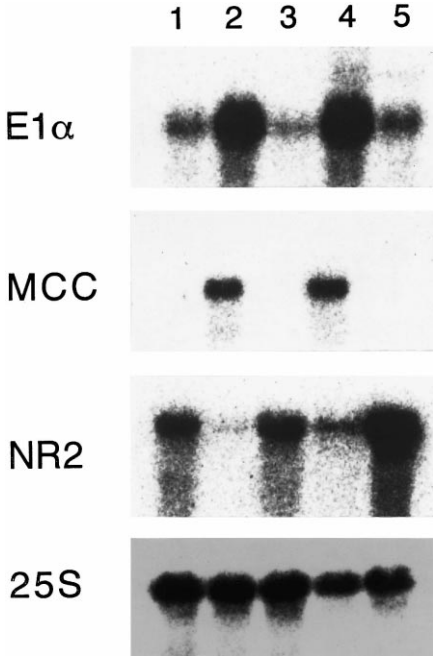

Fig. 2. Effects of sucrose and DCMU on the transcript levels of the E1α subunit gene. Detached leaves were floated on water for 48 h under continuous illumination (lanes 1, 4 and 5) or in the dark (lanes 2 and 3). Lanes 1 and 2, water without sucrose; lane 3, 3% sucrose; lane 4, 10 μM DCMU; lane 5, 10 μM DCMU plus 3% sucrose. 20 μg of RNA from each sample was used for RNA gel blot analysis. The biotinylated subunit gene of MCCase (MCC) was examined as a typical Leu catabolic enzyme. The transcript levels of the NR2 gene were examined as a sugar-inducible control.

3.2. Effects of sucrose and the photosynthesis inhibitor on the gene expression

Dark-adapted plants suffer sugar starvation because of the limitation in photosynthetic activity. To examine whether dark-induced expression of the E1α gene is caused by sugar deprivation, we examined the effect of sucrose and the photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on the E1α and MCCase transcripts (Fig. 2). When leaves were floated on a 3% (v/w) sucrose solution for 48 h in the dark, accumulation of the transcripts of the E1α and MCCase genes was totally prevented (lane 3). On the other hand, the transcript levels of the E1α and MCCase genes increased even under illumination when leaves were incubated with 10 μM DCMU, and the increase in the transcript levels in the presence of DCMU (lane 4) was canceled by addition of sucrose (lane 5). These results suggest that expression of the E1α and MCCase genes is induced by depletion of the photosynthetic products. We also examined the expression of NR2 as a sugar-inducible control. As shown in Fig. 2, the effects of sucrose and DCMU on the sugar-inducible NR2 gene were completely opposite to those on the sugar-repressible E1α and MCCase genes.

3.3. Expression of the E1α gene under sugar starvation in suspension-cultured cells

We used *Arabidopsis* suspension-cultured cells to further investigate the effects of sugars on the transcript levels of the E1α gene. Fig. 3A shows that sucrose deprivation triggered a marked accumulation of the E1α transcripts. In addition, accumulation of BCKDH proteins, as shown for the E2 subunit, occurred under this condition (Fig. 3B). We also found that MCCase transcripts accumulated in sugar-starved cells (Fig. 3A), which served as control in our experimental system, since MCCase activity has been found to increase in response to sugar starvation [17]. All of these results support our view that sugar starvation induces the expression of BCKDH to activate branched-chain amino acid catabolism [11,14].

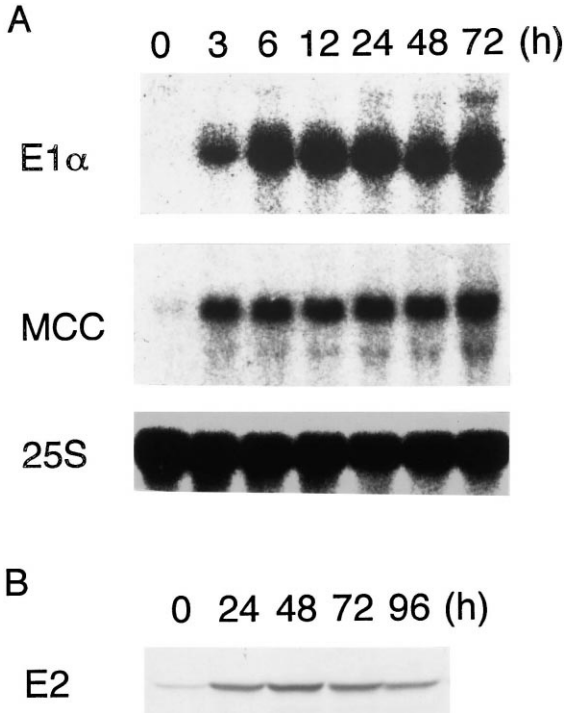

Fig. 3. Accumulation of the transcripts from the E1α subunit gene in *Arabidopsis* T87 suspension-cultured cells under sucrose starvation. RNA and soluble protein extracts were isolated from T87 cells that were incubated in a sucrose-free medium. A: 10 μg of RNA from each sample was used for RNA gel blot analysis. Results for the E1α subunit gene and the biotinylated subunit gene of MCCase (MCC) are shown. B: Soluble cell extracts (3 μg protein) were used for immunoblot analysis with antibodies raised against the E2 protein.

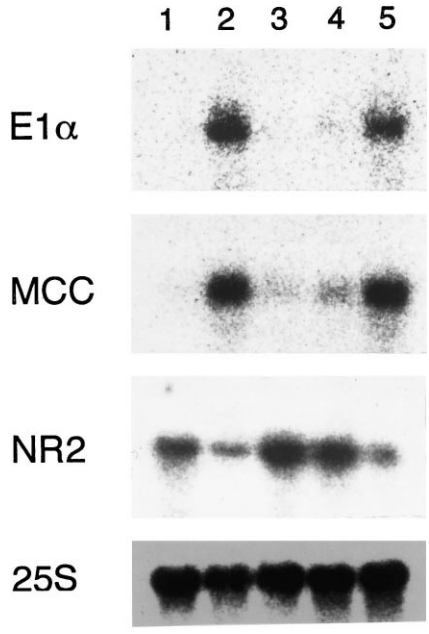

Fig. 4. Effects of glucose analogues on the transcript levels of the E1α subunit gene. T87 cells were incubated for 12 h with a fresh medium containing 10 mM glucose (lane 3), 0.5 mM 2-deoxyglucose (lane 4), or 10 mM 3-*O*-methylglucose (lane 5). Control cells were incubated with or without 2% sucrose (lanes 1 and 2). Each lane was loaded with 10 μg of RNA. Results for the E1α subunit and the biotinylated subunit of MCCase (MCC) are shown. Expression of a sugar-inducible NR gene was examined as control, which is likely to be regulated by hexokinase-mediated sugar signaling.

3.4. Effects of exogenous glucose analogues on the gene expression

We previously showed that 2-deoxyglucose, which is phosphorylated by hexokinase but is only slowly metabolized, rapidly repressed the expression of the E1β and E2 subunit genes, whereas 3-*O*-methylglucose, a non-phosphorylatable sugar, cannot suppress the gene expression [14]. Similarly, application of 0.5 mM 2-deoxyglucose but not of 10 mM 3-*O*-methylglucose to suspension cells prevented the accumulation of the E1α and MCCase transcripts (Fig. 4). As control, we showed that the sugar-inducible NR2 gene, which might be regulated by hexokinase-mediated sugar signaling (as shown for the NR1 gene [18]), was induced by 2-deoxyglucose but not by 3-*O*-methylglucose (Fig. 4). These results confirm that the phosphorylation of hexose by hexokinase is responsible for the sugar repression of each subunit gene of BCKDH and this is also the case with the MCCase biotinylated subunit gene.

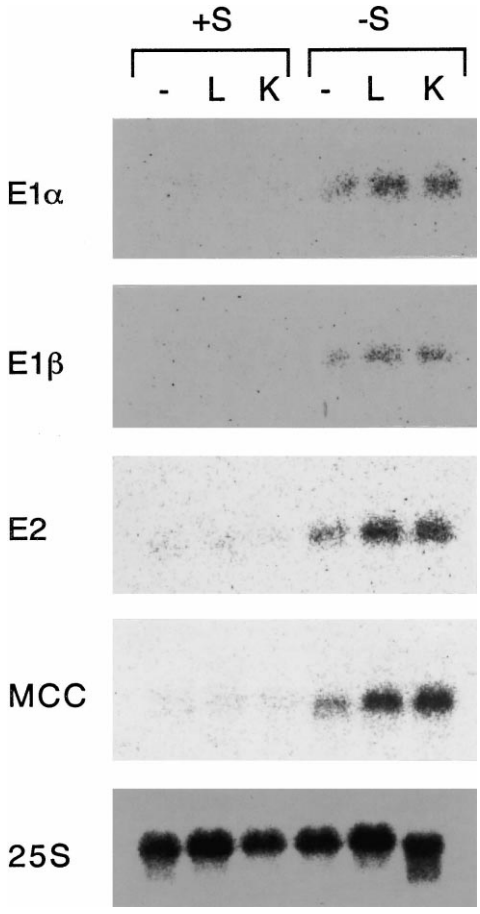

Fig. 5. Effects of Leu and KIC on the transcript levels of BCKDH and MCCase subunit genes. T87 cells were incubated for 6 h in the medium with (+S) or without (−S) sucrose, which contained 2 mM of Leu (L) or KIC (K). Each lane was loaded with 10 μg of RNA. Results for the E1α, E1β, and E2 subunit genes of BCKDH and for the biotinylated subunit gene of MCCase (MCC) are shown.

3.5. *Effects of Leu and KIC on the expression of genes for Leu degradation*

We investigated whether Leu and/or its derivative α-keto acid, KIC, regulates the gene expression involved in Leu degradation (Fig. 5). Quantitation of hybridization signals by radioluminography revealed that both Leu and KIC induced an approximately two-fold increase in the transcript levels of subunit genes for BCKDH and MCCase in sugar-starved suspension cells. Application of other amino acids, such as Asp or Gln (2 mM, pH 5.7), did not influence the expression patterns (data not shown). Leu and KIC were not able to enhance the gene expression in the presence of 2% sucrose, suggesting that Leu and KIC enhance the expression of genes for Leu degradation only when cells are starved for sugars.

4. Discussion

In this article, we found that accumulation of the transcripts from the BCKDH E1α subunit is induced by sugar starvation. Taken together with our report on other subunit genes, we provide evidence for the coordinated expression of each subunit gene of BCKDH under sugar starvation. Furthermore, accumulation of the transcripts from the BCKDH subunit genes actually resulted in the accumulation of its gene products, as evidenced by the E2 protein (this study), and accompanied by an increase in the enzymatic activity of BCKDH [11]. We also observed the coordinated expression of the biotinylated subunit of MCCase as well as that of BCKDH, both of which are located in the mitochondrial matrix and involved in branched-chain amino acid catabolism, under sugar-limiting conditions. These results support our proposal that BCKDH plays a regulatory role in the catabolism of branched-chain amino acids for respiratory uses.

Evidence for coordinated expression of BCKDH subunit genes has been obtained from mammals under certain condition [19], but not in other studies [20,21]. In our studies, a close correlation was found between expression patterns of the E1α, E1β, and E2 subunit genes in *Arabidopsis* ([11,14] and the present study), which may be important for stoichiometry in the assembly of subunits into a functional multienzyme complex. We found that sugar plays a key role in the coordinated expression. Phosphorylation of hexose by hexokinase, a sugar-sensory event in the expression of several sugar-modulated genes [14,18,22,23], is likely to be involved in the sugar suppression of the genes related to branched-chain amino acid catabolism ([14] and the present study).

We also found that Leu and KIC enhance the expression of subunit genes for BCKDH and MCCase under sugar starvation. This implies that branched-chain amino acids promotes their own catabolism when cells are starved for respiratory substrates. This idea is supported by the fact that addition of Leu to dark-adapted barley leaves elevated MCCase activity, which was involved in using Leu for respiratory purposes [24]. However, it should be noted that Leu and KIC were insufficient to trigger the accumulation of the transcripts from the genes for BCKDH and MCCase in the presence of sucrose. Similarly, addition of Leu to sugar-fed sycamore cells did not increase MCCase proteins [17]. Hence, it is likely that sugars are primarily responsible for the regulation of the expression of BCKDH and MCCase subunit genes, and that branched-chain amino acids efficiently enhance the gene expression only in sugar-starved cells. Since BCKDH catalyzes a rate-limiting step in the catabolic pathways of branched-chain amino acids, suppression of BCKDH genes by sugars possibly directs the flux of these amino acids to translocation and/or de novo protein synthesis [25]. By contrast, accumulation of branched-chain amino acids caused by sugar starvation [17,26] might fully activate BCKDH to use these amino acids as respiratory substrates. We therefore propose that regulation of BCKDH gene expression by sugars and Leu/KIC may have an important role in determining the metabolic fate of branched-chain amino acids, i.e. whether these amino acids should be used for protein synthesis or energy sources. Alternatively, by analogy to mammalian BCKDH as a scavenger of toxic branched-chain α-keto acids [3], sugar starvation-induced expression of BCKDH in plants might be involved in the disposal of excess amounts of branched-chain α-keto acids accumulated in sugar-starved cells.

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