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Metabolic Gene Expression in Lipid Metabolism during Cotyledon Development in Cucumbers and the Possibility of a Secondary Transport Route of Acetyl Units

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We investigated the expression of cucumber genes involved in lipid mobilization and metabolism during cotyledon development to compare gene activity and to study the direction of carbon (acetyl unit) transport between glyoxysomes and mitochondria. The core metabolic pathway involving 10 genes was examined in four intracellular compartments: glyoxysomes (peroxisomes), mitochondria, chloroplasts, and cytosol. Additionally, we tested the early germination response of dark-grown seedlings and the immediate light response for a further 3 days. According to the reverse transcription polymerase chain reaction (RT-PCR), 3-L-ketoacyl-CoA thiolase 2 (Thio2), isocitrate lyase (ICL), and malate synthase (MS), the genes involved in storage lipid mobilization showed a similar and consistent pattern of gene expression in seedling development. Furthermore, coordinate expression of the *A BOUT DE SOUFFLE* (*BOU*) gene with ICL and MS during seedling emergence pointed to a possible secondary route of acetyl unit (acetyl-CoA) transport between peroxisomes and mitochondria in cucumber. The expression of the *BOU* gene was light dependent, as shown by *BOU* activity in *Arabidopsis*, suggesting that the dark condition also results in weak membrane biogenesis. In addition, several genes were active throughout the development of the green cotyledon, even during senescence. In conclusion, this study summarizes oil-seed germination and gene expression during cucumber cotyledon development and proposes an additional route for acetyl unit transport.

Key words : BOU, cucumber cotyledon, gene expression, germination, lipid

Introduction

Cucumber seeds contain major stores of oil in the form of triacylglycerol (TAG), which supplies energy and carbon for seed germination and post-germinative growth. Mobilization of stored lipids is crucial for the seedling establishment in the oil-seed plants, such as in Cucurbitaceae and several other crop species [10, 12, 16]. TAG is converted into sucrose and then respired in germinating seedlings. The glyoxylate cycle and gluconeogenesis are of major importance in the oil-seed plants during germination and post-germinative growth. The first step in stored oil degradation is lipolysis of TAG into fatty acids through the action of several lipases. These fatty acids are then moved into specialized microbodies called glyoxysomes (or peroxisomes) together with coenzyme A (CoA) by transporting systems, forming an acyl-CoA that proceeds through β-oxidation to produce acetyl-CoA. Fatty acid β-oxidation occurs as a serial reaction with acyl-CoA oxidase, multifunctional proteins, and 3-ketoacyl-CoA thiolase (*Thio2* gene coding KAT) activity. It provides a carbon source for gluconeogenesis to synthesize sucrose in the cytosol and serves as substrates for cellular energy production in mitochondria [10]. Here, we examined cucumber *Thio2* gene expression, one of the enzymes of β-oxidation, for the initial stages of the lipid mobilization.

The β-oxidation product acetyl-CoA can be provided to either the glyoxylate cycle within the glyoxysome, or transported out to mitochondria for the tricarboxylic acid cycle (TCA cycle). First, two molecules of acetyl-CoA are required in the glyoxylate cycle: one molecule for the formation of malate, by malate synthase (MS), and the other molecule to form citrate, by glyoxysomal citrate synthase (Fig. 1). These components then follow a modified form of the respiratory TCA cycle and are finally routed into cytosol for gluconeo-

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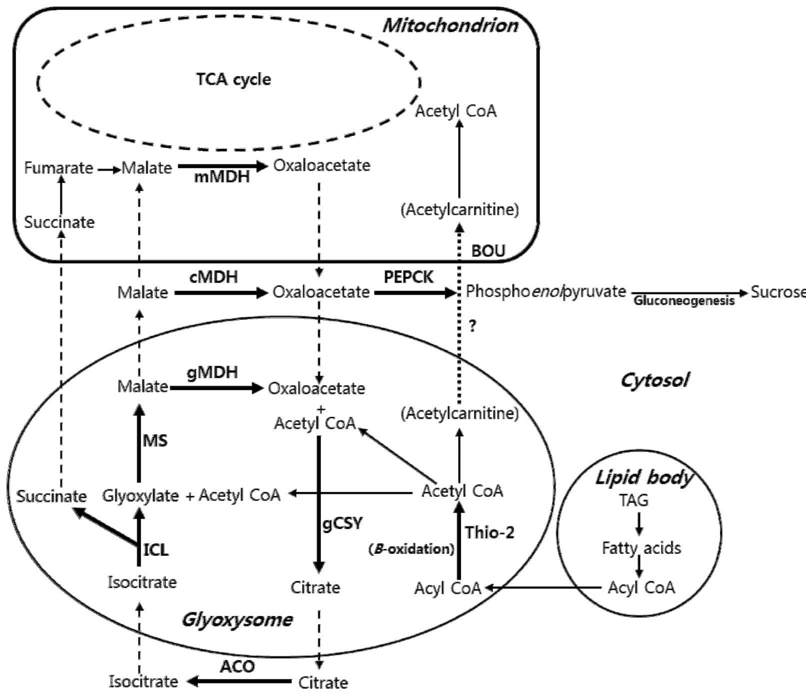

Fig. 1. Reactions of stored oil mobilization from the lipid body to cytosol and mitochondria in oil seed plants. Breakdown of TAG begins in the lipid body by an undefined pathway, with acyl-CoA moving to the glyoxysome. The specialized microbody, glyoxysome, contains both β-oxidation and glyoxylate cycle enzymes for catabolic mobilization of stored TAG. A suggested pathway by BOU may operate during cucumber germination for transport of the products of fatty acid β-oxidation from glyoxysomes to mitochondria. The glyoxylate cycle and major lipid mobilization reactions are marked with thick solid arrows. Possible transport of intermediates is marked by broken arrows.

genesis to produce sucrose. The five enzymes operating the glyoxylate cycle are glyoxysomal citrate synthase (gCSY), aconitase (ACO), isocitrate lyase (ICL), MS, and glyoxysomal malate dehydrogenase (gMDH) [8]. Except for cytosolic functional ACO, these key enzymes are located in glyoxysomes. As can be seen in Fig. 1, glyoxylate cycle operation requires import and export of the major metabolic intermediates, such as citrate, isocitrate, malate, and oxaloacetate across the glyoxysomal membrane. Secondly, a possible export system of acetyl-CoA to mitochondria through an acylcarnitine-like shuttle mechanism, *A BOUT DE SOUFFLE* (BOU), was proposed in *Arabidopsis* [9]. However, further reports have not yet been published regarding stored oil mobilization during seed germination.

We examined the gene expressions of glyoxylate cycle enzymes to compare the pattern of gene expressions throughout cotyledon development and early germination stages in the dark. Furthermore, one of the cucumber acylcarnitine carrier-like protein coding genes (*BOU1*) was examined to determine a secondary route of acetyl-CoA transport from peroxisome to mitochondrion in cucumber. Sequences of three predicted cucumber *BOU* genes (*BOU1*－*3*) were available in the National Center for Biotechnology Information (NCBI) database, by the recent cucumber genome sequencing. The *BOU1* gene shares 92% DNA sequence homology with *BOU2* and 89% with *BOU3* (data not shown). This gene provides a primary target for the potential existence of a direct shuttle system of acetyl-CoA in cucumbers. It would give additional genetic clues about whether BOU provides a primary or accessory route of carbon flow from peroxisome to mitochondrion during fatty acid β-oxidation under light conditions [9].

Materials and Methods

Plant material and growth conditions

Seeds of the cucumber *Cucumis sativus* L. were imbibed in sterile water at 4°C for 12 hr and sown in wet vermiculite. The resultant plants were maintained in a growth chamber (Jeio Tech, Korea) at 25°C and 70% humidity under continuous illumination. For the dark experiment, a growth chamber was maintained under the same conditions as the standard experiment, but without light. Cucumber cotyledons were collected exactly at 24 hr interval and immediately frozen in liquid nitrogen for total RNA extraction.

Total RNA preparation and estimation

An RNeasy Plant Mini Kit (Qiagen GmbH, Germany) was used to extract total RNA from cucumber cotyledon samples, and treated with deoxyribonuclease I (DNase I) (Qiagen GmBH) following the manufacturer's protocol. Three pairs of cotyledon were used in each stage of sampling for individual extraction. Final elution of total RNA was carried out twice in equal volumes (50 μl) of diethylpyrocarbonate-

(DEPC) treated sterile H_2O. The quality of total RNA was examined by 0.8% agarose gel electrophoresis in 0.5× Tris/Borate/EDTA (TBE) buffer at 40 V for 2 hr, and the quantity was examined at 260 nm through the BioPhotometer (Eppendorf, Germany). Total RNA extraction and estimation were carried out three times in each experiment at the single cotyledon level.

Semi-quantitative RT-PCR

An equal volume (5 µl) of total RNA solution was used to produce the first-strand of complimentary DNA (cDNA) by reverse transcription (RT) reaction. The poly(A)+ RNA was primed by the oligo (dT)18 primer using an AccuPower RT PreMix (Bioneer, Korea) following standard procedure. Polymerase chain reaction (PCR) was performed for 30 cycles using MyCycler (BioRad, USA) with an AccuPower PCR PreMix Kit (Bioneer, Korea) by gene-specific primers (5′-3′), as described in a previous report [6]. All primers were manufactured through Bioneer Co (Daejeon, Korea). An equal volume (10 µl) of RT-PCR products were fractionated on 1.0% (w/v) agarose gel in 0.5× TBE buffer for 60 min at 100 V, and the gel was digitally photographed and processed. These RT-PCR experiments were repeated at least three times independently under the same conditions.

Results

Germination of cucumber seeds in the dark

Cucumber seedling establishment and specific gene expression studies have been well documented under light conditions [4, 8]. In this epigeal germination, the cotyledons are brought over the ground due to fast elongation of the hypocotyl at around 60 hr after seed imbibition. During germination, the hypocotyl grows actively and becomes curved to bring the seed above the soil. After coming over the surface of the soil, the hypocotyl straightens and the loosened seed coats fall downward, after which the cotyledon becomes green. At this point, stored oil mobilization starts to be actively catabolized into carbohydrate through complex metabolic pathways (Fig. 1). However, cucumber seed germination and stored oil mobilization-related whole gene expression studies have not yet been performed under dark conditions. The beginning of cucumber seed germination did not differ between light and dark conditions, and the cotyledon emerged at around 60 hr after seed imbibition for both (Data not shown). After this, the cotyledon itself did not grow, and showed an intact oil reserve in the endosperm. However, the hypocotyl extensively grew up to 15 cm in the dark conditions (Fig. 2), but only 5 cm under light. This means that stored oil break down requires the light during early seedling development, as for normal growth and development in plants.

Total RNA changes during cotyledon development

The change of total RNA amount was dramatic at the single cotyledon level in the light-grown cotyledon, and sharply decreased after 5-7 days of seed imbibition (Fig. 3A). From this data, we prepared a single cotyledon level semi-quantitative RT-PCR experiment throughout cotyledon development and dark germination experiments. On the other hand, the total RNA amount was relatively stable in the dark-grown cotyledon (Fig. 3B). Following this, one-tenth of the volume of total RNA (5 µl) was adopted into first strand cDNA synthesis in each stage of cotyledon development.

Gene expression during cotyledon development

To determine the genes that express in germinating seeds and developing seedlings, gene-specific oligonucleotide primers were designed so that transcripts of each gene could

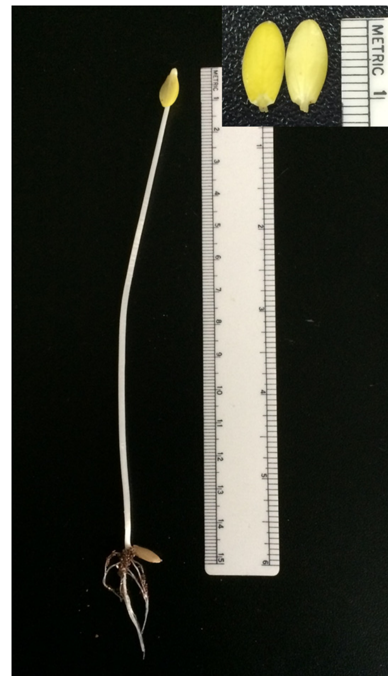

Fig. 2. This picture shows the dark germinating cucumber seedling 5 days after seed imbibition. The hypocotyl reached 15 cm in length and was 5 times longer than for the light grown seedling and cotyledon (see inset photo).

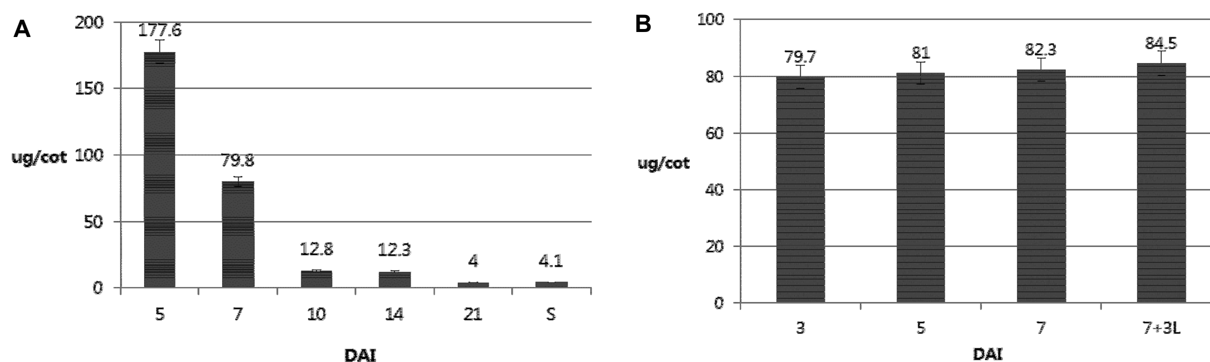

Fig. 3. RNeasy Plant Mini Kit extractable total RNA changes from a single cotyledon level (µg/cot) during development. Repeated three times. The black bar represents mean values. (A) Developmental changes in the light. (B) Dark germinating cotyledons with an additional 3 days light grown (7+ 3 L). DAI, days after seed imbibition; S, senescence (~50% yellow at mid-stage of senescing).

be detected by RT-PCR (Table 1). Therefore, we examined ten expressed genes that are essential to cucumber seed oil breakdown and mobilization during seedling establishment. The genes are *Thio2* for β-oxidation, five genes for the glyoxylate cycle, a gene for mitochondrial malate dehydrogenase (mMDH) that is responsible for the citric acid cycle, genes for cytosolic MDH and phospho*enol*pyruvate carboxykinase (PEPCK) that are responsible for gluconeogenesis, and finally the gene for acetyl-CoA transporting mitochondrial BOU (Table 1). DNA sequences and analysis tools were adopted from the NCBI database to obtain biochemical and genetic information that were used for PCR primer design for the tested genes (Table 1). The *Actin2* gene was used as a constitutive standard from a previous report [7]. As the single cotyledon level approach, equal volume of total RNA (5 µl) was used in the reverse transcription reaction for the developmental stages, and this was adopted throughout the experiment to compare relative changes in gene expression levels. As can be seen from the total RNA change in Fig. 3A, the RT-PCR result for *Actin2* gene expression is similar to that for the RNA changes in cotyledon development (Fig. 4). From these standard outcomes, we carried out RT-PCR for the ten of cucumber genes. Following RT-PCR results, gene expression patterns were divided into two classes. Firstly, constitutive expression group genes, which included *gCSY, ACO, gMDH, mMDH, cMDH,* and *PEPCK*. Secondly, germination expression group genes, which included *Thio2, ICL, MS,* and *BOU1*. Nevertheless, all genes showed strong expression at day 5 in germinating cotyledons, where the genes participate in stored oil break down, carbon mobilization, and gluconeogenesis. Most genes in the first group were expressed throughout cotyledon development, even in

Table 1. Cucumber genes and the gene-specific oligonucleotide sequences used in PCR with annealing temperatures

Gene name	Accession*	Primer Sequence (Forward/Reverse)	Annealing *Tm* °C**	(bp)
Actin2	AY338231	CTTGACTATGAGCAAGAACTCGAG / CCACTGAGGACAATGTTACCATAG	55	248
gCSY	XM_004143180	AAGCTGCCCTTTCAGATGAGT / TGTAGAAGGGGGCCTATCTCT	54	470
ACO	XM_004159154	ATGGAAGTGGAAGCTCTCGG / ACCTGCTGCTTATAGATCGC	52	524
ICL	Z35499	ATGAGCGATAAGCAAATGGAAGAG / AACTTGCTTTGCTTTTGCTCTTCA	58	411
MS	X15425	TTCGATCCATGAGACGTGAC / ATGGACAAAGACAGACTTACATTAC	52	572
gMDH	L31900	CCAAGTTCTTTCACACAAGAAGAGA / TGCCATCTCTTCAGCCTCTGATG	57	378
mMDH	XM_004137169	GCTCTCACCAAGCGAACACA / CAGTAAAGCCGGTTGCTCGT	56	473
cMDH	XM_004155147	TACCGAGTTTATTGCCACCGT / GGAGTAGAAGCATGGGAGAGT	52	525
PEPCK	AF481231	ATGGCTATCATGAGACATTGCTGA / GCTCTTAAAGGTCAACTTGACATA	52	443
Thio2	NM_001280761	TGTGGGTGTGGATCCTGCTA / AAGAGGGTGAGGGAAATGCC	56	547
BOU	XM_004168379	CAGACTGCAAGCTCAGAGTG / CTGGGACACTACGTGCCATA	52	449

*Accession codes from NCBI sequence database.
***Tm* values were calculated automatically by NCBI primer designing protocol (Pick Primer) (bp), Length of RT-PCR product.

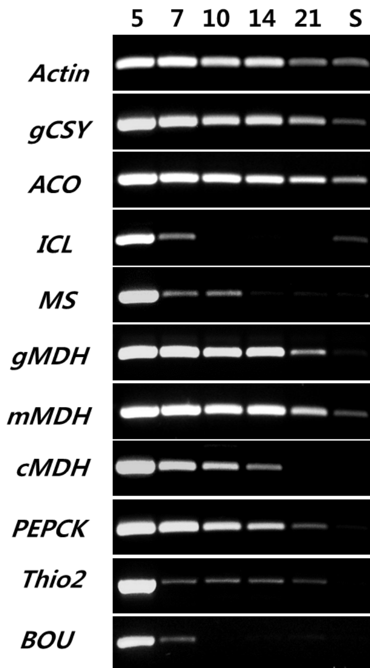

Fig. 4. Developmental changes of gene expression in the stored oil breakdown-related pathway by RT-PCR. Gene and enzyme names are as follows: *gCSY*, glyoxysomal citrate synthase; *ACO*, aconitase; *ICL*, isocitrate lyase; *MS*, malate synthase; *gMDH*, glyoxysomal malate dehydrogenase; mMDH, mitochondrial MDH; *cMDH*, cytosolic MDH; *PEPCK, phosphoenolpyruvate carboxykinase; Thio2*, 3-L-ketoacyl-CoA thiolase 2 (KAT2); *BOU*, acylcarnitine carrier-like enzyme. Numbers are DAI (days after seed imbibition) and S represents half-senescent cotyledons.

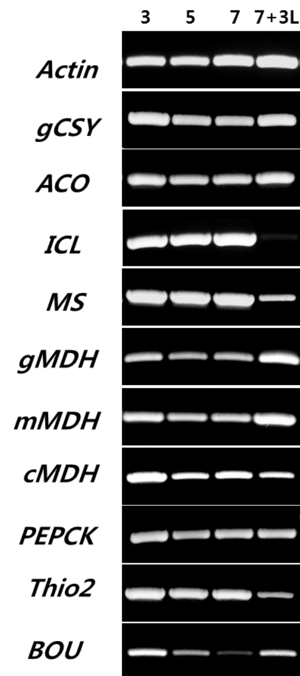

Fig. 5. RT-PCR results from dark-germinating cucumber cotyledons for examining gene expression changes. Cucumber seeds were imbibed in sterile water for 12 hr at 4°C, and then sown on wet vermiculite. Pots were put into the plant growth chamber in darkness at 25°C with 70% humidity. Cotyledons were collected in the dark from seedling stage and frozen in liquid nitrogen immediately for total RNA extraction. RT-PCR was carried out on a single cotyledon basis as per a previous developmental RT-PCR experiment. Numbers are DAI, and 7 + 3 L represents 7 days dark germination plus 3 days further light incubated cucumber cotyledons.

the senescence stage. However, the second group of genes showed strong expression only in the early stages (at day 5) of seed germination (Fig. 4). This reflects that they are specific and essential in stored oil breakdown and mobilization during cucumber seed germination. In particular, *ICL* and *BOU* gene expression patterns were almost identical throughout cotyledon development, except for a slight upregulation of the *ICL* gene in the senescence stage.

Gene expression in induced seedlings in the dark

To compare gene expression between light and dark conditions, seed germination was induced in the dark for up to 7 days. During dark germination, cotyledons were collected at 3, 5, and 7 days after seed imbibition. To determine the immediate light response, dark-grown seedlings were transferred to light conditions after 7 days and grown for a further 3 days. As can be seen in the dark germinated seedling at day 5 (Fig. 2), stored oils remained in the cotyledon. We tested reserved oils in crude extract from the day 5 dark-grown cotyledon using Sudan III reaction (Data not shown).

Most of the genes encoding enzymes for lipid breakdown and mobilization were highly expressed in dark germination up to 7 days after seed imbibition (Fig. 5). Organelle MDH genes (*gMDH* and *mMDH*) showed up-regulation in light (day DD7 + LD3) that might reflect the recovery of full metabolic systems under light illumination. However, the cucumber *BOU1* gene showed a different expression pattern, with the expression decreasing sharply at day 5, and barely detectable at day 7. Interestingly, cucumber BOU gene expression was recovered in the light (Fig. 5). It has been proposed that BOU activity is required for seedling establishment in the light, but not in the dark, as proposed in an earlier report [9]. We have examined that RT-PCR for three of predicted cucumber BOU genes revealed almost identical results between them (data not shown), and this finding is

consistent with BOU gene expression under light conditions. The possibility of a secondary or accessory transport system for acetyl units from acetyl-CoA via a carnitine shuttle is strongly implied, which is another pathway for fatty acid respiration in cucumbers. Furthermore, group 2 genes (*Thio2, ICL,* and *MS*) also showed a similar expression pattern in this experiment, where the genes are repressed in the light. It may also reflect that a similar gene expression regulation system can control group 2 gene activity during development.

Discussion

Cucumber seed germination and gene expression

TAG is a major carbon source, and is stored in oil bodies of endosperm cells in cucumber seeds. These stored nutrients in seeds will fuel post-germinative seedling establishment until photosynthesis can begin in the cotyledon. Initially, stored oil breakdown begins hydrolysis of TAG by lipases, forming fatty acids that are transported into peroxisomes by an, as yet, unconfirmed process [10]. Fatty acids are then esterified to acyl-CoA by long chain acyl-CoA synthetase. Subsequent β-oxidation produces acetyl-CoA in the glyoxysome by three catabolic steps. Here, we also showed that the most active expression of the *Thio2* gene during early seedling emergence was in the last step of β-oxidation, and this correlates well with findings from a previous report [13]. This result is also consistent with the period of most rapid fatty acid catabolism in cucumber cotyledons [10]. *Thio2* gene expression was prolonged in the dark for as long as the stored lipids remained in the endosperm. However, it also should be elucidated as to how *Thio2* gene expression is regulated by darkness. A possible suggestion may be that the acetyl-CoA accumulates in peroxisomes because the *Thio2* gene coding enzyme 3-L-ketoacyl-CoA thiolase (KAT) catalases the last step of fatty acid β-oxidation, which involves thiolytic cleavage of KAT to acyl-CoA (C_{n-2}) and acetyl-CoA (C_2).

Historically, ICL and MS are known as key enzymes in the glyoxylate cycle, because their enzyme activity and corresponding gene expression are highly specific in seed germination [4, 16]. Furthermore, *ICL* and *MS* gene expression has also demonstrated a slight up-regulation in cotyledon senescence [5]. In this report, we reconfirmed specific expression of *ICL* and *MS* genes during germination, but they showed a somewhat different pattern of gene expression in senescence. In the dark experiment, *ICL* and *MS* genes also showed similarity in gene expression until day 7, but the *MS* gene extended its activity under light conditions, and this is quite well matched with the activity extension seen in the developmental experiment. Therefore, it is believed that *ICL* and *MS* genes share gene expression control elements and factors. Three other glyoxylate cycle genes also revealed a high activity during germination, but this was more likely constitutive throughout cotyledon development. It may imply that the genes are not only specific to seed germination for stored oil mobilization, but are also involved in other metabolic processes such as anaplerotic reactions in autotrophic growing plant organs. In the dark experiment, *gCSY* and *ACO* gene activity was much higher than *gMDH* until day 7, but *gMDH* recovered levels under light conditions. The *gMDH* gene expression pattern is similar to that of the *mMDH* gene in both developmental and dark situations. However, the function of MDH is somewhat complex in metabolic pathways, because MDH enzymes participate in reversible reactions. Therefore, further metabolic function of the enzymes and gene expression regulation should be elucidated in future studies.

The glyoxylate cycle produces four carbon carbohydrates (malates) using the acetyl-CoA unit that comes from β-oxidation. These can be converted into hexose sugars by gluconeogenesis in the cytosol, and this is the final step of stored lipid mobilization in oilseed germination. This will lastly be consumed as fundamental energy for growth and development of the seedling. PEPCK is a key enzyme in gluconeogenesis, with the corresponding *PEPCK* gene showing not only a strong expression in germination, but it is also expressed throughout cotyledon development until senescence. Previously, we proposed that the cucumber *PEPCK* and *ICL* genes function in senescing the cotyledon for recycling of the demolished intracellular organelles and supply their nutrients to other parts of the cell [6, 8]. However, a different mechanism was proposed for ICL and PEPCK enzyme involvement in senescing organs that also needs to be elucidated in the future [1].

BOU expression and an alternative pathway

The stored oil mobilization process has been examined extensively, both genetically and biochemically, during plant germination for the last several decades. However, there is still much to be answered about the systemic functions of related enzymes and genes. The role of the glyoxylate cycle

and related enzymes have been reviewed in plant systems [2, 14]. A novel role for β-oxidation in seed dormancy is also currently proposed [3, 11].

Another possible route of acetyl-CoA has been proposed in Arabidopsis by mitochondrial BOU for postembryonic growth in the light as in yeast and animal systems [9]. Although, a purification of carnitine acetyl transferase (CAT) has also been reported from pumpkin mitochondria [15], and this was the first molecular genetic evidence of BOU in plant systems. However, no further report has since been made until the current study. Here, we report additional genetic evidence of BOU in plant systems through germinating cucumber cotyledons. According to our results, BOU may be the primary route of acetyl-CoA from peroxisomes to mitochondria during oilseed germination, because stored oil is immobilized without functional BOU in the dark, and no transport of acetyl-CoA occurs during β-oxidation. If so, the role of the glyoxylate cycle may not be the primary method of carbon transport from peroxisomes to mitochondria during germination.

Acknowledgment

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초록 : 오이 떡잎의 발달에서 지방 대사관련 유전자의 발현과 아세틸 단위체의 2차 경로 가능성

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본 연구는 떡잎의 발달 동안 지방의 유동 및 대사와 관련된 오이 유전자들의 발현을 조사하여 유전자의 활성을 비교하고자 하였으며, 글라이옥시좀과 미토콘드리아 사이의 탄소원(아세틸 단위)의 가능한 경로를 탐색하고자 하였다. 네 곳의 세포 내 소기관인 글라이옥시좀(퍼옥시좀), 미토콘드리아, 엽록체 및 세포질에서 작동하는 중요 대사경로의 10개 유전자들이 조사되었다. 나아가 암소에서 발아한 유식물체의 발아 초기 반응과 이후 3일간 빛을 주었을 때의 반응을 조사하였다. 역전사-중합효소연쇄반응(RT-PCR)에 따르면, 유식물체의 발달 동안에 저장지방의 유동과 관련된 *Thio2*, *ICL* 및 *MS* 유전자는 항상 유사한 유전자 발현 양상을 나타냈다. 오이의 발아 초기에 *BOU* 유전자와 함께 *ICL* 및 *MS* 유전자의 공조된 발현은 퍼옥시좀과 미톤콘드리아 사이에 아세틸 단위의 2차 통로의 존재 가능성에 대한 강한 증거이다. 앞서 보고된 연구에서 보여준 BOU 활성에서처럼 *BOU* 유전자는 빛 의존성으로 암소에서서는 세포막의 미약한 발달로 인하여 활성이 저하됨을 암시한다. 나머지의 유전자들은 떡잎이 초록색으로 발달하고 노쇠화 할 때까지 떡잎의 전 발달 기간 동안에 활성을 나타냈다. 본 연구에서서는 아세틸 단위의 운반에 대한 새로운 추가적 제안으로써 지방 저장 종자의 발아와 오이 떡잎의 발달과 관련된 유전자의 발현을 통해 처음으로 확인하였다.