

Re-Activation of the Expression of Glyoxysomal Genes in Green Plant Tissue

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Glyoxysomes are being replaced by leaf-type peroxisomes during the greening of dark-grown cucumber cotyledons. Light functions in this process as negative modulator of the gene expression of glyoxylate cycle enzymes but as positive regulator for the activation of glycollate oxidase formation. The differential gene expression was investigated at the level of mRNA amounts using cDNA probes hybridizing with malate synthase mRNA, isocitrate lyase mRNA, and glycollate oxidase mRNA. Hybridization probes were obtained from a cDNA library complementary to the germination-specific mRNAs of cucumber cotyledons. The process of replacement of glyoxysomal proteins by leaf peroxisomal proteins was reversed to a certain extent when greened cotyledons were brought back in the dark. Hybridization on Northern blots provided evidences that in greened cotyledons the amount of malate synthase mRNA and isocitrate lyase mRNA starts to increase upon dark treatment.

Introduction

Genes may be susceptible to activation by extracellular factors. Hormones produced within the organism, metabolites provided from outside, light or biotic signals bring about differential gene expression in a particular cell. As an example, proteins are synthesized preferentially and assembled within a differentiated form of an organelle because they take part in a metabolic process dominating in the cell at this stage [1–3].

Environmental and nutritional signals for gene control are transduced by plants and may lead to the formation of intracellular structures which are needed for special metabolic tasks. In cells of cucumber cotyledons, two such extreme metabolic situations are known. In the dark, the seedling grows heterotrophically and the organism depends on the extensive fat mobilization in the storage tissue which is in the cotyledon in this particular case [4, 5]. In the light, the cotyledons become green, and photoautotrophic growth based mainly on the role of chloroplasts is established [6, 7]. The gene activation required for the expression of the former function seems to be hormone regulated [8, 9] while the changes in gene activity preceding the as-

sembly of intracellular structures necessary for the later function are light-triggered processes [10].

Parallel to the change in metabolic function of cotyledon cells, the forms of microbodies in these cells are changed. According to their function, we distinguish between glyoxysomes [4, 5, 11] housed in dark-grown cotyledons and leaf peroxisomes which are characteristic of the fully greened cotyledons [11, 12].

Microbody proteins are encoded by nuclear genes and synthesized in the cytoplasm on free polysomes [11, 13]. Then, a presently unknown process follows by which the cytoplasmically made precursors are transferred into the organelle and assembled to mature enzymes by acquiring of co-factors and oligomerization. Experiments on gene expression at the level of enzyme activity and mRNA activity indicated that the control of microbody formation may primarily be at the transcriptional level [14]. We found that the composition of microbodies concerning their functional enzymes is virtually identical with the composition of the cytoplasmic pools of precursors destined for the import into the microbody [11, 15].

During the transition of glyoxysomes into leaf peroxisomes, which takes place during the first 50 h when dark-grown seedling were brought into light, the gene expression of glyoxysomal proteins is reduced [15, 16] while the one of leaf peroxisomal proteins is increased [17]. In this paper, we demonstrate this change in gene expression at the level of the amounts of individual mRNAs. In ad-

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dition, it is shown that this process can be reversed if light is eliminated and fat reserves are still present in the cotyledons.

Materials and Methods

Plant material, RNA isolation and translation in vitro

Cucumber seeds (*Cucumis sativus* L. Chinesische Schlangengurken) were germinated at 26 °C on sterilized vermiculite for 4 d. Seedlings were then placed under different light/dark regimes. White light of 10,000 lux was provided by fluorescent lamps. Cotyledons were used as the source of RNA as described previously [18, 19]. Poly(A)⁺ RNA was isolated by chromatography on oligo(dT)-cellulose. Subfractionation of poly(A)⁺ RNA was performed by electrophoresis on agarose gels containing methylmercuric hydroxide [18, 19].

Poly(A)⁺ RNA was subfractionated, extracted from the gel, purified by phenolization and precipitation, and used for the preparation of cDNA. Total RNA or poly(A)⁺ RNA was translated *in vitro* according to [18].

Preparation of a cucumber cDNA library in pBR 322 and characterization of clones

Double-stranded cDNA was prepared from poly(A)⁺ RNA, highly enriched in the size of 1.4–2.7 kb, according to [18, 19]. The cDNA was tailed with dC and annealed to dG-tailed pBR 322 at the PstI site. *E. coli* DH1 was transformed according to [19]. Tetracycline-resistant and ampicillin-sensitive colonies were grown on nitrocellulose. They were prepared for hybridization by the method of Grunstein and Hogness [20].

The cDNA bank was screened with various single-stranded cDNAs prepared from poly(A)⁺ RNA from cotyledons of etiolated seedlings or greening seedlings, from poly(A)⁺ RNA of green leaves, or from several poly(A)⁺ RNA subfractions obtained after electrophoretic isolation. Differential screening by colony hybridization yielded a number of clones coding for RNAs characteristic of the fat degrading stage of the cotyledons. In addition, the cDNA used for screening was enriched in sequences which encode proteins in the M_r range of 50,000–80,000. Plasmid DNA was isolated by the method of Birnboim and Doly [21] and

further purified by centrifugation in CsCl density gradients.

Final identification of clones was done by hybrid selection [22] of mRNA, *in vitro* translation, and immunoprecipitation of the respective proteins [18]. DNA sequence determination was carried out by the methods of Maxam and Gilbert [23] on various fragments of cDNA which were labelled at the 3'-end by T4-polymerase (3'-overhangs) or Klenow fragment of *E. coli* polymerase I (5'-overhangs). Alternatively, sequences were determined by the dideoxyribonucleotide method [24] after recloning in M13.

For probing glycollate oxidase mRNA, clone E 56 of a lentil cDNA library [18] was used.

Northern blot analysis

RNA was separated by electrophoresis on 1% agarose gels containing formaldehyde as described by Lehrach *et al.* [25]. Transfer to nitrocellulose membranes followed standard procedures [26]. The conditions described by [22] were used for hybridization with DNA labelled with [α -³²P]dCTP by using a multiprime kit. Dot blots were done according to [22].

Results

Preparation and screening of a cucumber cDNA library

A cDNA library enriched in sequences coding for proteins in the M_r range of 50,000–80,000 was constructed from poly(A)⁺ RNA prepared from cotyledons of etiolated cucumber seedlings. Lipid metabolism in cotyledons at this stage is generally associated with elevated levels of the mRNA activity for enzymes of the glyoxylate cycle and fatty acid β -oxidation (referred to as glyoxysomal enzymes). Thus, cotyledons of 4 d old seedlings are rich in mRNA coding for malate synthase and isocitrate lyase. Taking into account this enrichment, the initial screening of cDNA clones for germination-inducible genes was based on differential hybridization. cDNA from mRNA of cotyledons with fully expressed glyoxylate cycle, cDNA from mRNA of hypocotyls lacking the enzymes of glyoxylate cycle, cDNA from mRNA enriched by size separation, and cDNA derived from mRNA of green leaves were utilized for colony hybridiza-

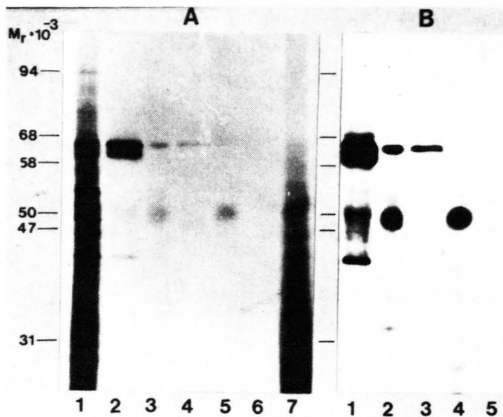


Fig. 1. Analysis of translation with hybrid-selected RNAs. cDNAs prepared from clones p7-56 and p6-74 were used to isolate specific mRNAs. Their activity, analyzed by translation *in vitro* in a reticulocyte lysate, yielded radioactive peptides (A; lane 1) which were characterized by their size by SDS gel electrophoresis. A, lane 2 and B, lane 1 have radioactive glyoxysomal proteins as internal standards. A, lane 3 and 4 are for isocitrate lyase mRNA selected with the p6-74 clone, prior to immunoprecipitation (lane 3) and afterwards (lane 4). B, lane 2 and 3 are for malate synthase mRNA selected with the p7-56 clone, prior to immunoprecipitation (lane 2) and afterwards (lane 3). Contamination with a 50,000 Da translation artefact (A, lane 5 and B, lane 4) was not present after immunoprecipitation. A, lane 6 and B, lane 5 are controls with preimmune serum. A, lane 7 contains proteins not precipitable with a mixture of anti-serum against isocitrate lyase and malate synthase.

tion. Twenty clones were selected which appeared to hybridize with cDNA characteristic of fat-degrading tissue and to encode proteins in the M_r range of approximately 60,000. Translation of hybrid-selected mRNA, followed by immunoprecipitation and electrophoresis (Fig. 1) revealed that two clones, p1-55 and p6-74, encode sequences of isocitrate lyase, and that one clone (p7-56) had sequences complementary to malate synthase mRNA. Clone p7-56 and p6-74 were further characterized by sequencing. A 200 bp sequence of clone p7-56 was almost identical with the C-terminal sequence for malate synthase published by Smith and Leaver [27]. The 1200 bp insert of p6-74 showed 75% homology with the isocitrate lyase cDNA prepared from castor bean mRNA [28]. The cDNA inserts of p7-56 and p6-74 were used as probes for determining the malate synthase mRNA and isocitrate lyase mRNA levels in cotyledons at different developmental stages.

Gene expression at the level of mRNA

The change in gene expression was analyzed by determining the pools of individual mRNAs. Northern blots of RNAs, prepared after exposing the seedlings the light for various times, were hybridized with the radioactively labelled inserts of p7-56 and p6-74. Malate synthase mRNA was characterized by a size of approximately 2.05 kb while the mRNA encoding isocitrate lyase was with 2.2 kb somewhat larger. We compared the levels of mRNAs coding for isocitrate lyase and malate synthase during the light-transition period.

During the beginning of the greening phase, the levels of these mRNAs were not significantly altered. Fig. 2 demonstrates that the level of isocitrate lyase mRNA starts to decline only after 18 h following the onset of light. After 36 h of light, however, the mRNA levels fell down to $\frac{1}{8}$ – $\frac{1}{6}$ of the values obtained with cotyledons left in the dark. Thus, the illumination time required for the reduction of stationary mRNA levels was probably close to 18 h, which is slightly shorter than the time-period characteristic for the decrease in synthesis of malate synthase and isocitrate lyase *in vivo* (unpublished results).

Comparison of the glyoxysomal enzyme mRNAs with the increase of glycollate oxidase mRNA reveals that the mRNA coding for a peroxisomal enzyme was present long before any reduction in the pools of glyoxysomal mRNAs was detectable. As demonstrated in Fig. 2, exposure to light for 36 h is sufficient to reduce the level of

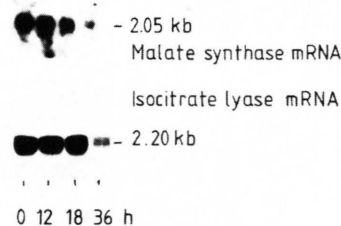


Fig. 2. Decrease in the level of malate synthase mRNA and isocitrate lyase mRNA in cotyledons during the illumination of 4 d old dark-grown seedling. The autoradiography of respective parts of Northern blots shows signals at the position of mRNA visualized by hybridization with labelled cDNA probes. The four lanes contain the RNAs prepared from cotyledons which were illuminated for the times indicated.

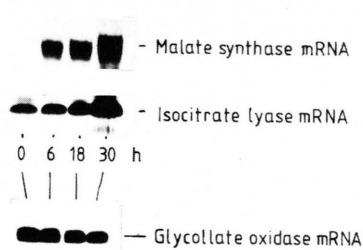


Fig. 3. Increase in the amount of malate synthase mRNA and isocitrate lyase mRNA various times after the transfer of greened cotyledons into the dark. The intensities of the signals are proportional to the amount of mRNA present in the respective total RNA preparation. Only the parts of the Northern blots are shown which encompass the positions of the respective mRNAs. The times indicated at the four lanes represent the length of dark period. Besides labelled malate synthase cDNA and isocitrate lyase cDNA, also glycollate oxidase cDNA was used as hybridization probe.

gene expression for glyoxysomal function to a low level. At that time, the cotyledons were greened (100 µg chlorophyll per cotyledon) but still contained some lipid reserves (1.3 mg triglyceride per cotyledon). Transfer of these plants into the dark and analysis of mRNAs after various times in the dark demonstrated that darkness in the presence of lipid effects the reactivation of the genes encoding malate synthase and isocitrate lyase (Fig. 3). This is in contrast to other green tissues, *e.g.* leaves, where darkness does not bring about the expression of glyoxysomal functions.

Discussion

An increasing amount of data support the hypothesis that in cells at different developmental stages, the microbody proteins are assembled according to the set of genes who are activated at that time [11, 13]. The results are consistent with

the assumption that mainly transcriptional control, but not import or other posttranslational processes, are relevant for the protein composition of the various microbody species. Therefore, an increase in the levels of mRNAs coding for glyoxysomal proteins are taken as first signs of glyoxysomal functions. This is a sensitive means to detect the time point where the alteration in the functions of peroxisomes begins.

On this basis, we compare in the light regime the expression of glycollate oxidase which takes less than 3 h [18] and the reduction of glyoxysomal gene activity which starts after 18 h (Fig. 2). In the dark, the re-activation of genes encoding glyoxysomal enzymes needs less than 30 h (Fig. 3) which is a shorter period of time than the long lag period of glyoxysome formation observed during the early stage of germination in the dark. We have argued [18] that the velocity of this kind of gene expression may be somehow controlled by the space of microbodies present in the cell.

The increase in isocitrate mRNA level and malate synthase mRNA level in a photosynthetic tissue has not been observed till now. However, low levels of enzyme activity of isocitrate lyase or malate synthase have been detected in leaves under senescent conditions [29, 30]. As for the susceptibility to activation of glyoxysomal genes, it is probable that not auxins make the difference between greened cucumber cotyledons and leaves. Rather the nutritional control as it is also observed in yeasts growing on oleate [31] may be the decisive signal.

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