Plant Science 272 (2018) 157–163

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Regulation of expression of the mitochondrial and peroxisomal forms of citrate synthase in maize during germination and in response to light

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ARTICLE INFO

Keywords:
Zea mays L.
Citrate synthase
Mitochondria
Peroxisomes
Phytochrome
Promoter methylation

ABSTRACT

Expression of genes encoding the mitochondrial and peroxisomal forms of citrate synthase (EC 2.3.3.1) was studied in maize (*Zea mays* L.) in scutella during germination and in leaves depending on light regime. During germination, citrate synthase activity increased in scutella both in mitochondria and in fatty-acid metabolizing peroxisomes (glyoxysomes) by day 6 and then declined. This was preceded by the peak of expression of the genes encoding the mitochondrial (*Csy1*) and peroxisomal (*Csy2*) forms of citrate synthase occurring on the day 3 of germination, after which the expression of *Csy1* gradually and of *Csy2* sharply declined. The decrease of expression of both genes was followed by the increase of promoter methylation which was more intensive for the gene encoding the mitochondrial form. In leaves, the activity of the mitochondrial form was much higher than that of the peroxisomal form and increased in darkness, while the peroxisomal form was almost undetectable in darkness and increased in the light. The mitochondrial form was inhibited by white and red light while the peroxisomal form was induced by white, red and blue light indicating the involvement of phytochrome and cryptochrome. The mechanism of light regulation of citrate synthase involved promoter methylation leading to the inhibition of corresponding genes and exhibiting opposite patterns for *Csy1* and *Csy2*. Citrate synthase was purified from mitochondria and glyoxysomes of maize scutellum. The mitochondrial form had higher optimum pH as compared to the glyoxysomal form and possessed higher affinity to oxaloacetate and acetyl-CoA. It is concluded that expression of citrate synthase during germination and in response to light is regulated by methylation of promoters of corresponding genes.

1. Introduction

Citrate synthase (CS; EC 2.3.3.1, formerly 4.1.3.7) catalyzes condensation of the acetyl group of acetyl-CoA with oxaloacetate (OAA) forming citrate. It represents the first step of the tricarboxylic acid (TCA) and glyoxylate cycles. It is localized in the mitochondrial matrix as well as in glyoxysomes which are the specialized peroxisomes performing β-oxidation of fatty acids and the glyoxylate cycle. In Arabidopsis, three genes (*At2g42790, At3g58740* and *At2g58750*) encode the peroxisomal CS and two genes (*At2g44350* and *At3g60100*) encode the mitochondrial CS. In addition and in relation to its role in the TCA and glyoxylate cycles, CS is involved in several specific processes associated with the reaction that it catalyzes. They include citrate formation with following excretion from roots for the facilitation of uptake of mineral nutrients [1,2], citrate accumulation during ripening of fruits [3], citrate production for the cytosol with its further metabolic transformation for nitrate and ammonium assimilation [4,5] and for the formation of other organic acids [6].

The mitochondrial CS is the best characterized form in plant and animal cells [7,8]. It represents an important regulatory point of metabolism as the enzyme is inhibited by ATP and high redox level, acetyl-CoA, succinyl-CoA, and citrate [7,8]. Redox regulation of citrate synthase includes the reduction/oxidation of cysteine residues mediated by thioredoxin [9]. The peroxisomal (glyoxysomal) CS is important for operation of the glyoxylate cycle through which oxidation of fatty acids is linked to gluconeogenesis as well as to fatty acid respiration involving the peroxisomal step that provides the exit route from fatty acid metabolism in oilseeds and supplies the formed citrate to the TCA cycle in mitochondria [10,11]. It has been demonstrated that the glyoxysomal CS is synthesized as a precursor of higher molecular-mass [12] on the endoplasmic reticulum and then imported to the glyoxysomal peroxisome [13,14].

Contrary to the mitochondrial form, the glyoxysomal CS is not or less inhibited by ATP [15–17]. Although CS is the most abundant in the

Abbreviations: CS, citrate synthase; DTNB, 5,5-dithiobis (2-nitrobenzoate); MS-PCR, methyl-specific polymerase chain reaction; OAA, oxaloacetate; TCA cycle, tricarboxylic acid cycle

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https://doi.org/10.1016/j.plantsci.2018.04.017
Received 14 March 2018; Received in revised form 16 April 2018; Accepted 18 April 2018
Available online 21 April 2018
0168-9452/

glyoxysomal type of peroxisomes [18–20], its presence was demonstrated in the non-glyoxysomal peroxisomes [21]. Its role there may be related not only to a limited oxidation of fatty acids followed by the conversion of formed acetyl-CoA in the glyoxylate cycle which increases during the aging of leaves [22] but also to the supply of citrate to cytosol for several processes including the initiation of the pathway of mevalonic acid formation which is a universal precursor of isoprenoids [23].

While the expression of both CS forms in scutella during germination is connected with the metabolic function of this organ in degradation and utilization of stored fats [19], in photosynthetic tissues citrate synthase expression is related to the regulation of respiration by light and to citrate supply for metabolic processes. The involvement of phytochrome mechanism in regulation of CS in plants has been shown in several studies. In earlier works it was revealed that light via the phytochrome mechanism induces the transition of glyoxysomes to leaf peroxisomes with the suppression of synthesis of key glyoxysomal enzymes including citrate synthase [24,25], at the same time synthesis of the mitochondrial citrate synthase is stimulated by light [26]. The microarray data of Thum et al. [27] and Tepperman et al. [28,29] demonstrate repression of the peroxisomal gene (*At2g42790*) and induction of the mitochondrial gene (*At2g44350*) for citrate synthase in Arabidopsis, indicating the enhanced efflux of citrate from phytochrome-activated mitochondria [30], while the peroxisomal formation of citrate is suppressed.

The role of DNA methylation in regulation of expression of the genes encoding the enzymes of energy metabolism and, in particular, the TCA cycle enzymes received attention only recently [31–33]. Previous research in our lab has revealed that expression of genes encoding subunits A and B of succinate dehydrogenase in maize is regulated by methylation of their promoters [34,35]. The effect of DNA methylation on regulation of the genes having a CpG island in the promoter is achieved via chromatin condensation which prevents binding of transcription factors [36–38], and, even if the promoter does not contain CpG island, methylation of specifically located cytosines can still result in attenuation of binding [39].

In the present study we examined expression of two genes annotated in GenBank (*Csy1* and *Csy2*) encoding correspondingly the mitochondrial and peroxisomal forms of CS, during germination of maize seeds in scutella and in the leaves of maize seedlings depending on light regime. The CS molecular forms from mitochondria and glyoxysomes were purified and characterized, their kinetic properties determined. Our study demonstrates that expression of the genes encoding both forms is controlled by promoter methylation, and that light exhibits opposite effects on the expression of the mitochondrial and peroxisomal form of CS in leaves.

2. Materials and methods

2.1. Object of investigation

Scutella of germinating seeds and leaves of 14-day old seedlings of maize (*Zea mays* L., cv. Voronezhskaya-76) were used in the experiments. For the study of CS expression and activity in scutella during germination, maize seeds were soaked in water and germinated in darkness at 25 °C on two layers of filter paper in Petri dishes. Scutella were collected every 48 h during 10 days after the start of experiment. For the study of CS expression and activity in green leaves, germinating seeds were transferred on the 5th day to the controlled conditions with 12 h photoperiod with light intensity of 25 W m^{-2} and temperature 22 °C.

2.2. Irradiation by red, far red and blue light

The 14-day old maize plants were placed in dark chamber for 24 h and then irradiated for 15 min by red, far red, and blue light of the intensity 0.044 W m^{-2}, using correspondingly the light diodes of 640–680 nm (KIPD40M40-K-Pb, Russia) for red light treatment, 710–750 nm (ZL127A-5, Russia) for far red light treatment, and 465–470 nm (Proton, Russia) for blue light treatment. The samples were collected 3 h after irradiation. The plants incubated in darkness for 24 h were used as a control. Light-incubated plants were harvested after 6 h of illumination provided by luminescent lamps.

2.3. Preparation of extracts and separation of organelles

For extraction of total citrate synthase activity 1 g of scutellum or leaf tissue was homogenized in 5 ml of grinding medium (100 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA and 0.01% Tween 80), filtered through four layers of cheesecloth and centrifuged at 12,000*g* for 10 min, the supernatant was used for activity measurements. For separation of mitochondria and peroxisomes, 2 g of maize scutellum or leaves were homogenized in 10 ml of 100 mM Tris-HCl buffer, pH 7.6, containing 0.3 M sucrose and 1 mM EDTA and filtered through four layers of cheesecloth. After centrifugation at 1300 g for 5 min, the debris of cell walls was discarded and supernatant was centrifuged again at 14,000*g* for 20 min. The pellet containing mitochondria and peroxisomes was resuspended in the same buffer and used for further separation of organelles. Separation of organelles was performed by isopycnic centrifugation in the density gradient of sucrose at 25,000*g* for 4 h on the ultracentrifuge Beckman (USA). The step gradient consisting of 2.5 M, 2.3 M, 1.8 M, 1.5 M and 1.3 M of sucrose was used. For identification of organelles the activities of marker enzymes were measured: succinate dehydrogenase for mitochondria, catalase for peroxisomes, and lactate dehydrogenase for cytosol [19]. The cross-contamination between mitochondria and glyoxysomes was 6–8% in all experiments.

2.4. Determination of citrate synthase activity and kinetic studies

CS activity was measured spectrophotometrically at 412 nm via detection of the formation of thionitrobenzoate anion [40,41]. The medium contained 100 mM Tris-HCl (pH 8.0), 0.1 mM 5,5-dithiobis (2-nitrobenzoate) (DTNB), 0.5 mM OAA, and 0.2 mM acetyl-CoA. The values of *K*m were determined from the double reciprocal plots [42]. The effect of pH on CS activity was studied in 100 mM Tris-HCl buffer containing various concentrations of either OAA or acetyl-CoA at the fixed saturated concentration of the second substrate, in the range of pH 7–9. The distribution of CS between mitochondria and glyoxysomes in scutellum during germination (Fig. 1) was calculated relative to the values of total CS activity and of total protein content determined according to Lowry et al. [43].

2.5. Purification of the mitochondrial and cytosolic forms of citrate synthase

The mitochondrial and peroxisomal forms of CS were purified from the fractions of mitochondria and peroxisomes obtained after the isopycnic centrifugation. The organelles were ruptured in the 25 mM Tris-HCl buffer, pH 8.0, containing 0.01% Tween 80, and fractionated by ammonium sulfate (40–70% saturation for the mitochondrial form and 50–70% for the peroxisomal form). The pellet after centrifugation for 15 min at 15,000*g* was dissolved in the same buffer and loaded on the column of Sephadex G-25 (1.5 × 20 cm). The eluate containing active fractions with CS activity was loaded on the column of DEAE Sephacel (Toyo Soda, Japan) and eluted by the linear gradient of KCl (0–300 mM). The obtained active fractions were collected and further purified by gel chromatography on the Sephadex G-200 column (2 × 40 cm).