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Short communication

Expression and promoter methylation of succinate dehydrogenase and fumarase genes in maize under anoxic conditions

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ABSTRACT

Succinate dehydrogenase (SDH) and fumarase enzyme activity and expression of genes encoding the SDH subunits A (*Sdh1-2*), B (*Sdh2-3*), C (*Sdh3*), D (*Sdh4*) and the mitochondrial (*Fum1*) and cytosolic (*Fum2*) isoforms of fumarase were quantified in maize (*Zea mays* L.) seedlings exposed to atmospheres of air (control), N_2, and CO_2. The catalytic activity of SDH gradually declined in plants exposed to N_2 atmospheres, with ~40% activity remaining after 24 h. In seedlings incubated in CO_2, the suppression was even more pronounced. Fumarase activity was more stable, decreasing by one third after 24 h of anoxia. The level of *Sdh1-2* transcripts in seedlings declined significantly under N_2 and even more rapidly upon exposure to CO_2, with a concomitant increase in methylation of the corresponding promoters. The level of *Sdh2-3* and *Sdh3* transcripts also decreased under N_2 and CO_2, but the changes in promoter methylation were less pronounced, whereas the changes in the level of *Sdh4* expression and promoter methylation were minor. Expression of *Fum1* and *Fum2* was affected by N_2 and CO_2 atmospheres, however without changes in corresponding promoter methylation. It is concluded that, under conditions of oxygen deficiency, succinate accumulates mainly due to downregulation of SDH gene expression and reduction of enzyme activity, and to a lesser extent due to the decrease of fumarase gene expression.

1. Introduction

In the conditions of oxygen depletion metabolic pathways are reorganized to meet energy requirements for hypoxic plant cells. Oxygen deficiency results in the limitation of operation of mitochondria and switching to fermentation pathways leading to formation of ethanol, lactate and alanine (Gibbs and Greenway, 2003). Mitochondria can keep functionality due to their operation with the alternative electron acceptor (nitrite) which leads to nitric oxide (NO) formation, its scavenging by phytoglobin, and nitrate reduction (Igamberdiev and Hill, 2004; Gupta and Igamberdiev, 2011). Fermentation pathways and the phytoglobin/NO cycle support a limited rate of ATP formation (Stoimenova et al., 2007), also pyrophosphate becomes an important alternative energy currency (Igamberdiev and Kleczkowski, 2011).

Operation of the tricarboxylic acid (TCA) cycle is altered under hypoxic conditions and switched to a non-cyclic mode (Rocha et al., 2010; António et al., 2016) but in a different way than in photosynthetic tissues in the light when citrate is used as a source of 2-oxoglutarate for glutamate formation (Sweetlove et al., 2010; Igamberdiev et al., 2014; Igamberdiev and Eprintsev, 2016). In both cases, succinate dehydrogenase (SDH) is inhibited but, while in the light this results in accumulation of malate and fumarate (Igamberdiev et al., 2014 and references therein), under anoxia glutamate is actively decarboxylated in the cytosol to form γ-aminobutyric acid (GABA), which is further metabolized to succinate in mitochondria. Succinate accumulation under low oxygen was demonstrated in early studies (Effer and Ranson, 1967; Zemlyanukhin and Makeev, 1969) and confirmed in recent works (Rocha et al., 2010; Narsai et al., 2011; António et al., 2016; Behr et al., 2017). It was shown that this accumulation could be due to the inhibition of SDH (Kennedy et al., 1987; Fox and Kennedy 1991; Chirkova et al., 1995) and fumarase (Couée et al., 1992).

Inhibition of succinate oxidation under anoxia was reported in the early work of Ranson et al. (1957) and linked to the accumulation of CO_2. A direct suppression of SDH by elevated CO_2 was reported in soybeans (Gonzalez-Meler et al., 1996). However, the mechanisms of this suppression have not been established to date. In the previous studies we demonstrated that SDH inhibition in the light is mediated by changes in the level of methylation of promoters of the genes encoding the subunits A and B (Eprintsev et al., 2016a), occurs through the action of phytochrome (Popov et al., 2010) and involves calcium elevation in the nucleus (Eprintsev et al., 2013). The inhibition of fumarase

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expression in the light also takes place via phytochrome and calcium (Eprintsev et al., 2016b). In the current study we report the results indicating downregulation of the expression of SDH and fumarase genes in the conditions of oxygen deficiency. We have chosen the gene *Sdh1-2* for the subunit A and *Sdh2-3* for the subunit B because these genes were markedly expressed in leaves, while the other genes encoding these subunits are active mainly during germination (Popov et al., 2010; Eprintsev et al., 2016a). We show that while downregulation of SDH expression can be partly controlled epigenetically via methylation of promoters of the gene encoding the subunit A, the decrease in expression of fumarase genes does not involve promoter methylation.

2. Methods

Maize (*Zea mays* L., cv. Voronezhskaya-76) plants were grown hydroponically at 22 °C and light of intensity of 25 W m^{-2} 14 h per day. Green leaves of 14-day-old plants were used for the experiments. For measuring SDH and fumarase activities, maize leaves were homogenized on ice with 50 mM Tris–HCl buffer, pH 7.5, containing 5 mM $MgCl_2$, 1 mM EDTA, 2 mM cysteine and 0.01% Tween 80. The samples were centrifuged at 15,000*g* for 10 min at 4 °C and the supernatant was desalted on a Sephadex G-10 column. The total protein was assayed by the method of Lowry et al. (1951).

SDH activity was assayed by the decrease of optical density at 600 nm (Igamberdiev and Falaleeva, 1994; Popov et al., 2010) using the T70+ UV–vis Spectrophotometer (PG Instruments Limited, UK). The medium contained 50 mM potassium-phosphate buffer, pH 7.8, 0.1 mM phenazine methosulphate, 0.08 mM dichlorophenolindophenol (DCPIP), 2 mM sodium azide, and 10 mM sodium succinate. The activity was calculated using the extinction coefficient of the oxidized DCPIP 21 $mM^{-1}cm^{-1}$, and the unit of activity was determined as the amount of enzyme reducing 1 μmol DCPIP in 1 min at 25 °C. Fumarase activity was measured by an increase in the optical density at 240 nm due to the formation of a double bond in fumarate molecule (Eprintsev et al., 2014). The assay medium contained 50 mM tris-HCl buffer, pH 8.0, 50 mM malate and 3 mM $MgSO_4$. The extinction coefficient of fumarate 2.44 mM^{-1} cm^{-1} (Moore and Proudlove, 1983) was used, and the unit of activity was taken as the amount of enzyme producing 1 μmol of fumarate in 1 min at 25 °C.

Total RNA extract from maize leaves was isolated by the phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Reverse transcription of mRNA was performed using the reverse transcriptase M-MuLV (SibEnzyme, Russia) according to the instruction manual. PCR with gene-specific primers was conducted using the set of reagents Amplisens (Helicon, Russia). The primers for PCR analysis had the following sequences: for *Sdh1-2:* forward – 5′-CAAACGGGTCACTTCCAACT-3′, reverse – 5′-CCAAAACTGTCCCACGTCTT-3′, для *Sdh2-3:* forward – 5′-GAGAGGCTACAGGCAATAACTGAG-3′, reverse – 5′-AGTTTTAAACATTGATTCTTATTG-3′, for *Sdh3:* forward – 5′-AAGGAGGCTTCTCCATCTCC-3′, reverse – 5′-CAGAGCTGCTACAGGGGAAG-3′, for *Sdh4:* forward – 5′-TTCGGCCATTACGGTCCGGAAG-3′, reverse – 5′-AGCGGAATGAAATCTTGAGGA-3′, for *Fum1:* forward – 5′-GATTACTTCGATCATTGAGGT-3′, reverse – 5′-ACCAGAACTCGCGGATGTGGC-3′, for *Fum2:* direct – 5′-ACAAACTTGCCATTCGTCACC-3′, reverse – 5′-TGGTTCATTCTCAGGCAGAGA-3′.

Real-time PCR was performed using the LightCycler96 (Roche, Switzerland) using Green I as SYBR dye. The gene of elongation factor ef-1α (Nicot et al., 2005) was used as a reference. The relative level of expression of the studied genes was quantified by the $2^{-\Delta\Delta Ct}$-method (Livak and Schmittgen, 2001).

DNA was extracted using the kit DNA-sorb-S (Institute of Epidemiology, Russia) according to the instruction manual. DNA modification by sodium bisulfite was performed according to Warnecke et al. (2002). The original sample of DNA (50 μg) was denatured by 0.3 M NaOH and deaminated using 4.15 M sodium bisulfite solution and 0.2 M hydroquinone solution at pH 5.0. The resulting sample was desalted using QIAEX® II Gel Extraction Kit (150) (QIAEX, Germany). Desulfonation was performed by 0.3 M NaOH followed by neutralization using 10 M ammonium acetate and 96% ethanol at pH 5.6. The following PCR reaction with methylation-specific primers was conducted using the kit AmpliSence (Helicon, Russia). The PCR reaction was performed on Tertsik PCR machine (DNA-Technology, Russia). For the selection of primers for methylation-specific PCR (MS-PCR) the programme MethPrimer – Li Lab, UCSF (http://www.urogene.org/methprimer/index1.html) was used (Supplementary Table 1).

The quantitation of MS-PCR was performed on the basis of the results of electrophoregrams of PCR products. The values of the degree of promoter methylation are the cumulative results of PCR analysis of the studied CG-dinucleotides in the promoter of each specific gene. Since there could be three types of result (non-methylated, partially methylated and fully methylated), the numerical calculation was performed in the following way. The value 0% was assigned when all three studied CG-dinucleotides are non-methylated; 25% – one or two CG-dinucleotides are partially methylated; 50% – one or two CG-dinucleotides are methylated; 75% – one or two CG-dinucleotides are partially methylated and the remaining are fully methylated; 100% – all three CG-dinucleotides are methylated (Eprintsev et al., 2012, 2016a).

The experiments were performed in 3–4 replications with three repeats. The obtained results were evaluated using the Student test. Significant differences at $P < 0.05$ are discussed. On the figures the average values and their standard deviations are presented.

3. Results

3.1. The effects of N_2 and CO_2 atmosphere on succinate dehydrogenase and fumarase activities

Exposition of maize plants to anoxic N_2 and CO_2 atmosphere resulted in marked changes of the activities of SDH and fumarase (Fig. 1). In N_2 atmosphere the decline of SDH was smooth decreasing slightly

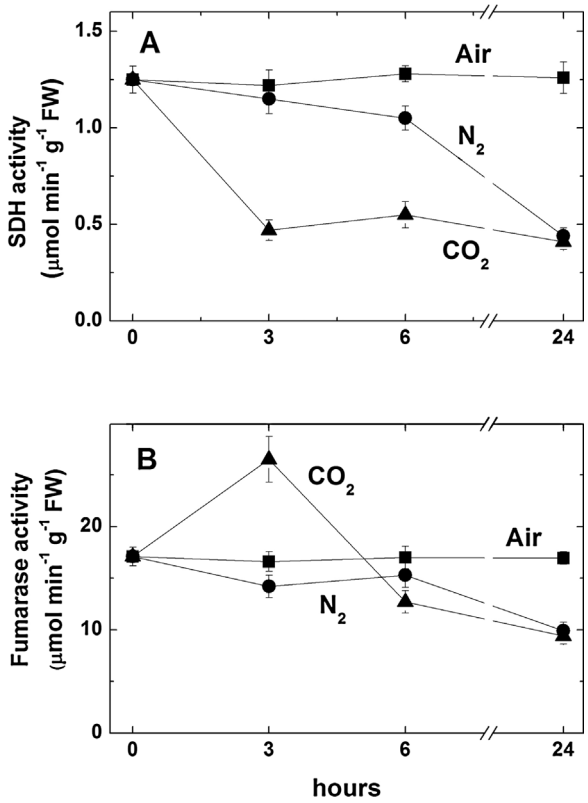

Fig. 1. Catalytic activities of succinate dehydrogenase (A) and fumarase (B) in leaves of maize plants exposed to air, N_2 and CO_2.