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Short communication

Expression and properties of the mitochondrial and cytosolic forms of fumarase in sunflower cotyledons

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ABSTRACT

Fumarase (EC 4.2.1.2) is encoded in sunflower (*Helianthus annuus* L.) by two genes (*FUM1* and *FUM2*) expressing correspondingly the mitochondrial and the cytosolic form. Both forms have been purified from sunflower cotyledons and characterized. Three quarters of fumarase activity is located in the mitochondrial and one quarter in the cytosolic fraction. The cytosolic form has lower pH optimum than the mitochondrial form, it possesses higher affinity to malate, activated by Mn^{2+} and less efficiently by Mg^{2+} while the mitochondrial form is activated only by Mg^{2+}. It is proposed that the mitochondrial form is involved in the respiratory processes linked to the tricarboxylic acid cycle and the cytosolic form participates in the utilization of succinate produced in the glyoxylate cycle providing the flux to gluconeogenesis in germinating sunflower seeds.

1. Introduction

Fumarase (fumarate hydratase; EC 4.2.1.2) interconverts malate and fumarate and participates in the tricarboxylic acid cycle. In addition to the mitochondrial form, fumarase was detected in the cytosol in Arabidopsis (Pracharoenwattana et al., 2010) and maize (Eprintsev et al., 2014). Both forms of fumarase are encoded by separate genes. Some plants, like poplar (Tuskan et al., 2006) and tomato (Mueller et al., 2008), contain only one fumarase gene. While in Arabidopsis the function of cytosolic fumarase was attributed to nitrogen accumulation and to cold acclimation (Dyson et al., 2016), in maize scutellum its participation in utilization of succinate formed in the glyoxylate cycle was demonstrated (Eprintsev et al., 2014). Participation of both forms of fumarase during mobilization of stored lipids and their conversion to carbohydrates may also be essential for germinating oily seeds of dicotyledonous plants, although such studies were not performed so far.

Sunflower is an important agricultural plant accumulating lipids in seeds and converting them to carbohydrates via β-oxidation of fatty acids and glyoxylate cycle (Schnarrenberger et al., 1971). Sunflower accumulates significant amounts of fumarate both in shoots and in roots exceeding the amounts of malate and citrate (Saber et al., 1999). Fumarate conversion during germination of sunflower seeds supplies malate not only for respiratory reactions of the tricarboxylic acid cycle but also for its conversion to carbohydrates via gluconeogenesis. Fumarate has multiple functions in plants (Araújo et al., 2011; Igamberdiev and Eprintsev, 2016) including storage of carbon (Zell et al., 2010; Zubimendi et al., 2018) and modulation of synthesis and consumption of malate during photosynthesis (Igamberdiev et al., 2001; Arias et al., 2013).

Previously (Eprintsev et al., 2014) we investigated expression and properties of the mitochondrial and cytosolic fumarase in maize scutellum, studied phytochrome-mediated regulation of fumarase by light in Arabidopsis (Eprintsev et al., 2016) and its regulation by low oxygen in maize (Eprintsev et al., 2017). The current study presents the data on expression and activity of the mitochondrial and cytosolic forms of fumarase in sunflower cotyledons. Properties of both forms were determined on the purified preparations and compared to the properties of the mitochondrial and cytosolic forms of fumarase from maize. Participation of both forms in the tricarboxylic acid cycle and in the utilization of succinate formed in the glyoxylate cycle is discussed.

2. Methods

Cotyledons of germinating seeds of sunflower (*Helianthus annuus* L., cv. Flagman) were used. Seeds were germinated on moist filter paper and transferred for hydroponic growth on the second day at 12 h daylight of 25 W m^{-2} and temperature 25 °C.

Fumarase activity was measured spectrophotometrically at 240 nm by detecting the formation of the double bond of fumarate as described earlier (Eprintsev et al., 2014). The extinction coefficient of fumarate 2.44 mM^{-1} cm^{-1} was used and the amount of enzyme producing 1 μmol of fumarate in 1 min was taken as a unit of enzyme activity. The

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Table 1
Purification of the mitochondrial and cytosolic forms of fumarase from sunflower cotyledons (n > 3, p < 0.05).

Stage		Volume ml	Protein mg	Activity μmol min^{-1}	Specific activity μmol $min^{-1}mg^{-1}$	Yield %	Purification
Homogenate		7.9	98.6	10.2	0.1	100	1
Fractionation by $(NH_4)_2SO_4$ (20–60%)		2.5	19.7	7.81	0.39	76.5	3.9
Gel filtration on Sephadex G-25		3.5	19.3	7.92	0.41	77.6	4.1
Ion exchange chromatography on DEAE-Sephacel	Mitochondrial form	1.8	0.72	4.3	5.97	42.15	59.7
	Cytosolic form	1.7	0.57	3.1	5.43	30.4	54.3

A

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                    ++++++++++++++++++++++++++++++++++++++++++ ++++++++++++ +++++
SEQ1             1  AAAGAAACCAATTGGGCCGAGGCCCATACCTCTCGCTGAAAGAAGTCAGCGTCAAACCCA
FUM1_H.annuus    1  AAAGAAACCAATTGGGCCGAGGCCCATACCTCTCGCTGAAAAAAGTCAGCGTCAGACCCA

                     +++++++ ++++++++++++++++++++++++++++++++++++++++++ ++++++++++++
SEQ1             61  GGGTGCTCATACTTTTTTACTTCTCTTTTCTCCAGCCATCTCGGGGCACACACACCCTTC
FUM1_H.annuus    61  GGGTGCTTATACTTTTTTACTTCTCTTTTCTCCAGCCATCTCGGGGCATACACACCCTTC

                      +++++++++++++++ +++++++++
SEQ1            121  TGCAATTCATTACAGTCATTCCGG-----------------------------------
FUM1_H.annuus   121  TGCAATTCATTACATTCATTCCGGttaatttgtaggaaaaagaaatggcgatggttcaag
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B

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                    ++++ ++++++++ +++++++  ++++++++++++ +  ++ ++ + + ++++++++++++
SEQ2               1  AGATAACAGTCTT-AAATGATCTATTCCCAACCGTGACTCAGAT-GTATCTACAATATTG
FUM2_H.annuus    580  AGATCACAGTCTTCAAATGATACATTCCCAACCGTTATGCATATCGCAGCTACAATATTG

                    ++++++++++ ++++++++ ++++++++++++++++++++++++++++++ +++++++
SEQ2               59  GAAATGGCAATTTATTTTACTAATCCCTTCAATATTGTGATATTATTACTTGATCTAAA
FUM2_H.annuus     640  GAAATGGCAACTTATTTTATTAATCCCTTCAATATTGTGATATTATTACTTAATCTAAA
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Fig. 1. Nucleotide sequences of two amplicons and corresponding fragments of fumarase genes from *Helianthus annuus*. (**A**) Alignment of the fragment of *H. annuus* gene *FUM1 (LOC110864574*; Accession Number XM_022113675.1*)*, abbreviated as FUM1_H. annuus, and of the 121-bp amplicon (SEQ1); (**B**) Alignment of the fragment of *H. annuus* gene *FUM2 (LOC110944702*; Accession Number XM_022186349.1*)*, abbreviated as FUM2_H. annuus, and of the 117-bp amplicon (SEQ2).

total protein was determined by the method of Lowry et al. (1951).

Polyacrylamide gel electrophoresis was conducted according to Davis (1964). The specific staining was performed in 50 mM tris-glycine buffer, pH 7.2, containing 5 mM $MgCl_2$, 10 mM sodium fumarate, 400 μg ml^{-1} nitrotetrazolium blue, 40 μg ml^{-1} phenazine methosulfate, 2 mM NAD^+ and 1.5 units of malate dehydrogenase (Sigma-Aldrich, St. Louis, MO) (Worsfold et al., 1977; Heeb and Gabriel, 1984). For protein staining, the silver method (Shevchenko et al., 1996) was used.

The study of subcellular localization of fumarase was performed on the days 3 and 7 of germination using differential centrifugation of cotyledon extract (Eprintsev et al., 2014). Lactate dehydrogenase was used as a marker of cytosol and succinate dehydrogenase was a marker of mitochondria.

Purification of the two forms of fumarase was performed from the total extract of cotyledons using ammonium sulfate fractionation (20–60%), gel filtration of Sephadex G-25 and ion-exchange chromatography on DEAE Sephacel using 30–150 mM linear gradient of KCl.

The Michaelis constants were determined from the double reciprocal plots using malate as a substrate (Eprintsev et al., 2014). The medium was 50 mM potassium phosphate buffer (pH 7.5 for the cytosolic and 8.0 for the mitochondrial form) containing 5 mM $MgCl_2$ and various concentrations of sodium malate. The effect of pH on fumarase activity was studied in 50 mM potassium phosphate buffer containing 5 mM $MgCl_2$ and 10 mM malate. The influence of metal ions on enzyme activity was determined at pH 7.5 for the cytosolic and 8.0 for the mitochondrial form by adding KCl, $CaCl_2$, $MgCl_2$ or $MnCl_2$ at 5 mM concentration.

RNA isolation was performed as described earlier for maize (Eprintsev et al., 2014). Specific primers for fumarase genes were developed on the basis of the sequences of fumarase genes from *H. annuus*: for the *FUM1* gene, forward 5′-CCATACCTCTCGCTGAAAGAA-3′, reverse 5′-ATTGCAGAAGGGTGTGTGTG-3′, and for the *FUM2* gene, forward 5′-AAATGATCTATTCCCAACCGTGAC-3′, reverse 5′-TCACAAT ATTGAAGGGATTAGTAAA-3'. The PCR analysis of cDNA with gene-specific primers after separation by electrophoresis in 1% agarose gel showed only one band for each pair of primers. This confirms the specific binding of primers with the cDNA. The specificity of primers was confirmed also by re-amplification of the PCR product extracted from the gel (not shown).

All experiments were repeated three times with analytic assays taken also three times. The data on figures are means of three biological repeats ± SD. The table of purification (Table 1) represents the data of a typical experiment repeated four times. The statistically significant differences at P < 0.05 are discussed.

3. Results

3.1. Identification of two genes

Sequencing of the purified nucleotide bands from the agarose gel and their comparison with the GenBank database revealed that the amplicons had significant homology with mRNA of fumarase genes from *H. annuus* (Fig. 1). For the 121 bp amplicon, the identity with the *FUM1* gene (*LOC110864574*; accession Number XM_022113675.1) of *H. annuus* was 97%. The comparison of nucleotide sequences of the *FUM2* gene (*LOC110944702*; accession Number XM_022186349.1) of *H. annuus* encoding the cytoplasmic fumarase and of the 117 bp amplicon showed 88% identity (Fig. 1). This confirms that in the course of amplification, using degenerate primers, the amplicons of mRNA of two genes having homology with nucleotide sequences of two fumarase genes of *H. annuus* annotated in the GenBank were obtained.

The PCR analysis of cDNA with gene-specific primers after separation by electrophoresis in 1% agarose gel showed only one band for each pair of primers (Fig. 2A). This confirms the specific binding of