Plant Physiology and Biochemistry 97 (2015) 264–271

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

Characterization of maize spermine synthase 1 (ZmSPMS1): Evidence for dimerization and intracellular location

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ARTICLE INFO

Article history:
Received 21 August 2015
Received in revised form
6 October 2015
Accepted 10 October 2015
Available online xxx

Keywords:
Bimolecular fluorescence complementation
Yeast two-hybrid
Spermine synthase
C-terminal
Homodimer
Subcellular localization
Spermine synthesis

ABSTRACT

Polyamines are ubiquitous positively charged metabolites that play an important role in wide fundamental cellular processes; because of their importance, the homeostasis of these amines is tightly regulated. Spermine synthase catalyzes the formation of polyamine spermine, which is necessary for growth and development in higher eukaryotes. Previously, we reported a stress inducible spermine synthase 1 (*ZmSPMS1*) gene from maize. The ZmSPMS1 enzyme differs from their dicot orthologous by a C-terminal extension, which contains a degradation PEST sequence involved in its turnover. Herein, we demonstrate that ZmSPMS1 protein interacts with itself in split yeast two-hybrid (Y2H) assays. A Bimolecular Fluorescence Complementation (BiFC) assay revealed that ZmSPMS1 homodimer has a cytoplasmic localization. In order to gain a better understanding about ZmSPMS1 interaction, two deletion constructs of ZmSPMS1 protein were obtained. The ΔN-ZmSPMS1 version, where the first 74 N-terminal amino acids were eliminated, showed reduced capability of dimer formation, whereas the ΔC-ZmSPMS1 version, lacking the last 40 C-terminal residues, dramatically abated the ZmSPMS1-ZmSPMS1 protein interaction. Recombinant protein expression in *Escherichia coli* of ZmSPMS1 derived versions revealed that deletion of its N-terminal domain affected the spermine biosynthesis, whereas C-terminal ZmSPMS1 truncated version fail to generate this polyamine. These data suggest that N- and C-terminal domains of ZmSPMS1 play a role in a functional homodimer.

1. Introduction

Polyamines (PAs) are ubiquitous molecules that have wide-ranging function in a number of physiological processes (Handa and Mattoo, 2010). The complexity of their metabolism and the fact that PAs homeostasis is tightly regulated support the idea that PAs are essential to cell survival (Moinard et al., 2005). In eukaryotes, the most abundant PAs are putrescine (Put), and the higher order PAs, such as spermidine (Spd) and spermine (Spm), all of them with specific biological functions. Moreover, in archaea, bacteria and plants, a structural isomer of spermine termed thermospermine (TSpm) has been found (Knott et al., 2007). Spd, Spm, and TSpm are synthesized by the action of aminopropyl transferase enzymes, spermidine synthase (SPDS; EC 2.5.1.16), which converts Put into Spd and spermine synthase (SPMS; EC 2.5.1.22) or thermospermine synthase (TSPMS, ACL5; EC 2.5.1.79), that transforms Spd into Spm or TSpm, respectively. The aminopropyl transferases use decarboxylated *S*-adenosylmethionine as aminopropyl donor. Because of their positive charge, PAs can bind to anionic molecules, such as DNA, RNA, proteins, and chromatin; thus, PAs are implicated in many critical cellular processes including cell division, differentiation, replication, transcription, translation, enzymatic activity alterations, membrane stabilization, and the functioning of certain ion channels (Igarashi and Kashiwagi, 2000; Childs et al., 2003; Kusano et al., 2008; Zepeda-Jazo et al., 2011).

In particular, Spm may function as a free radical scavenger, protecting DNA from free radical attack (Ha et al., 1998). In mammals, Spm synthesis is essential for normal growth and development (Wang et al., 2004). In *Arabidopsis thaliana*, the *AtSPMS* gene is

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http://dx.doi.org/10.1016/j.plaphy.2015.10.017
0981-9428/

required for protection against drought and salt stress, which is related to the role of Spm in gating inward rectifying potassium channels (Yamaguchi et al., 2006, 2007). It is known that Spm homeostasis is regulated at biosynthesis level by the activity of the SPMS enzyme, and through the flavin-containing polyamine oxidases that catalyzes oxidative deamination of Spm (Guerrero-González, et al., 2014; Ahou et al., 2014). In this regard, the aminopropyl transferase dimerization has been related to enzyme activity and protein stability (Korolev et al., 2002; Wu et al., 2008).

In previous research work, we characterized under salt stress the *Zea mays* spermidine synthase *ZmSPDS2* and *ZmSPDS1* genes (Jiménez-Bremont et al., 2007). Moreover, *in silico* and yeast functional complementation analyses revealed that *ZmSPDS2* gene, initially classified as spermidine synthase, encode a spermine synthase that we named as ZmSPMS1 (Rodríguez-Kessler and Jiménez-Bremont, 2008; Rodríguez-Kessler et al., 2010). This reclassified ZmSPMS1 enzyme differs from their dicot orthologous by an extension of 31 C-terminal residues, which is conserved among monocot enzymes (Rodríguez-Kessler and Jiménez-Bremont, 2008), it stretch encodes a protein degradation PEST sequence (region enriched in proline, glutamic acid, serine, and threonine) (Rogers et al., 1986). We found that the ZmSPMS1 PEST sequence leads to specific degradation of the β-glucoronidase (GUS) reporter protein by 26S proteasome, suggesting that this proteolytic sequence could regulate the ZmSPMS1 turnover (Maruri-López et al., 2014).

In this study, we explored the protein dimerization of ZmSPMS1 using split-ubiquitin yeast two-hybrid (Y2H) system, and then confirmed by Bimolecular Fluorescence Complementation (BiFC) assay in *Nicotiana benthamiana* leaves. In order to determine the segments involved in the ZmSPMS1-ZmSPMS1 protein interaction, we designed two N- and C-terminal truncated versions derived from full-length ZmSPMS1 protein. We observed that the absence of either N- and C-terminal domains compromise the dimer assembly in Y2H and BiFC approaches. Finally, the capacity to produce Spm of ZmSPMS1 derived versions was analyzed in *Escherichia coli* expression system. These results reveal that ZmSPMS1 regions involved in dimer formation also affect its activity.

2. Materials and methods

2.1. Yeast strains and medium

For the yeast two-hybrid split-ubiquitin analysis we employed the *Saccharomyces cerevisiae* NMY51 parental strain. Transformed yeast cells were selected on synthetic dextrose SD medium, containing yeast nitrogen base without amino acids (6.7 g/L YNB, 0.6 g/L dropout mix, and 20 g/L glucose). The yeast auxotrophies were supplemented as required with amino acids and nitrogenous bases [0.1 g/L leucine, 0.02 g/L tryptophan, 0.02 g/L histidine (LWH), 2 mg/L uracil (U), and 0.01 g/L adenine (A)]. Two percent of agar was added to prepare solid medium.

2.2. Plant material

For the BiFC approach we used *N. benthamiana* seeds, which were sown on a mix of 50% vermiculite and 50% soil and grown for three to four weeks in controlled greenhouse conditions under long-day photoperiod cycles (16 h light/8 h dark) at 22 °C ± 1 °C.

2.3. Split-ubiquitin constructs

The *ZmSPMS1* open reading frame (1170 bp) was PCR amplified from pYES-*ZmSPMS1*-V1 vector (Rodríguez-Kessler et al., 2010) by Phusion High-fidelity DNA polymerase (Thermo Scientific, Carlsbad, CA, USA). The PCR product was digested with *Sfi*I enzyme, and the generated cohesive fragment was cloned in-frame into DUALhunter vectors pDHB1-Cub and pPR3N-NubG. To determine the regions involved in the ZmSPMS1 protein interaction, we generated by PCR two N- and C-terminal deletion versions derived from full-length ZmSPMS1 protein. The ΔN-ZmSPMS1 version was constructed by removing their 74 N-terminal residues. In the ΔC-ZmSPMS1 construct, we deleted the 40 C-terminal amino acids. These constructs were PCR amplified and directionally cloned into the DUALhunter (pDHB1-Cub and pPR3N-NubG) vectors digested with *Sfi*I enzyme. All constructs were confirmed by DNA sequencing.

2.4. Yeast two-hybrid analysis

The protein interaction between ZmSPMS1 constructs were examined in yeast using the DUALhunter kit (Dualsystems Biotech, Schlieren, Switzerland), according to the manufacturer's protocol. To confirm correct expression and functionality of the system, each of the bait constructs (ZmSPMS1 full-length, ΔN-ZmSPMS1, ΔC-ZmSPMS1) were co-transformed with prey control vectors, which express a fusion of endogenous ER protein Alg5 with the wild-type NubI (positive) portion or with the NubG (negative) portion bearing the isoleucine to glycine mutation. The ZmSPMS1 constructs were co-transformed with pPR3N and grown on SD-LWHA medium supplemented with 0.1, 0.5, and 1 mM 3-aminotriazole (3-AT, a competitive inhibitor of the *HIS3* gene product) to determine the concentration required for testing the protein interaction ZmSPMS1-ZmSPMS1. In the DUALhunter system, protein interaction leads to activation of the *lacZ, HIS3, and ADE2* reporter genes. In this sense, each bait construct was co-transformed with each prey construct in the *S. cerevisiae* NMY51 strain, plated on SD medium without Leu and Trp (SD-LW, to select for transformed yeast), and grown at 28 °C for 4 days. For the screen interaction, transformed yeast cells were grown on SD liquid medium without LWHA to an OD_{600} of 0.8. Five microliters of serial dilutions (1:10, 1:100, 1:1000, and 1:10,000) were spotted on solid SD-LWHA medium supplemented with 0.1, 0.5, and 1 mM 3-AT, and grown for 4 days at 28 °C. As a positive interaction control we co-transformed yeast cells with SV40 LargeT antigen (-Cub) and tumor suppressor p53 (-NubG) vectors (Dualsystems Biotech). These experiments were repeated three times with similar results.

2.5. β-Galactosidase activity assays

The β-galactosidase enzyme activity was analyzed qualitatively by the X-gal assay (Möckli and Auerbach, 2004). Yeast strains expressing the different ZmSPMS1 interactions were selected and inoculated into 5 mL of each SD-LW and SD-LWHA liquid medium supplemented with 0.1 mM 3-AT. The yeast cells were grown overnight to an OD_{600} of 0.8. Subsequently, 1 mL of each culture was centrifuged at 3000 rpm for 5 min. The supernatant was decanted and cell lysis was carried out by two cycles of freeze-thaw (3 min in liquid nitrogen and then 3 min at 37 °C). Finally, the pellets were suspended in 20 μL of distilled water, transferred to a 96 well plate and mixed with 100 μL of PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) containing: 500 μg/mL of X-gal and 0.05% (v/v) β-mercaptoethanol. The samples were incubated at room temperature and the enzyme activity was monitored for blue color development after 30 min.

The quantitative O-nitrophenyl β-D-galactopyranoside (ONPG) assay, was conducted with 0.01 mL of protein yeast transformant extracts added to final volume of 0.1 mL of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 38.3 mM 2-mercaptoethanol, pH 7) supplemented with ONPG substrate