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Research article

A maize spermine synthase 1 PEST sequence fused to the GUS reporter protein facilitates proteolytic degradation

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ABSTRACT

Polyamines are low molecular weight aliphatic compounds involved in various biochemical, cellular and physiological processes in all organisms. In plants, genes involved in polyamine biosynthesis and catabolism are regulated at transcriptional, translational, and posttranslational level. In this research, we focused on the characterization of a PEST sequence (rich in proline, glutamic acid, serine, and threonine) of the maize spermine synthase 1 (ZmSPMS1). To this aim, 123 bp encoding 40 amino acids of the C-terminal region of the ZmSPMS1 enzyme containing the PEST sequence were fused to the *GUS* reporter gene. This fusion was evaluated in *Arabidopsis thaliana* transgenic lines and onion monolayers transient expression system. The ZmSPMS1 PEST sequence leads to specific degradation of the GUS reporter protein. It is suggested that the 26S proteasome may be involved in GUS::PEST fusion degradation in both onion and Arabidopsis. The PEST sequences appear to be present in plant spermine synthases, mainly in monocots.

1. Introduction

Spermidine and spermine synthase (SPDS; EC 2.5.1.16 and SPMS; EC 2.5.1.22) belong to a class of aminopropyl transferase enzymes that uses decarboxylated *S*-adenosylmethionine (dcSAM) as substrate for the transfer of an aminopropyl group to a diamine or triamine acceptor molecule producing the higher polyamines (PAs) spermidine (Spd) and spermine (Spm), respectively (Rodríguez-Kessler et al., 2010; Shao et al., 2012). Because of their positive charge, PAs can bind to anionic molecules, such as DNA, RNA, proteins, and chromatin. PAs act as regulatory molecules in various cellular processes including cell division, synthesis of DNA and proteins, gene expression, 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).

Due to these multiple functions, PA homeostasis is crucial and must be strictly maintained by regulation of their biosynthesis, catabolism, conjugation, and transport (Wallace et al., 2003; Carbonell and Blazquez, 2009; Mattoo et al., 2010). In particular, the biosynthesis of these polycations is controlled at transcriptional, posttranscriptional, translational, and posttranslational level (Malmberg and Cellino, 1994; Zhang et al., 2003; Rodríguez-Kessler et al., 2006; Ivanov et al., 2009; Takahashi and Kakehi, 2010).

In mammals, intracellular levels of PAs are maintained through proteolytic degradation of the first biosynthetic enzyme ornithine decarboxylase (ODC; EC 4.1.1. 17), which catalyzes putrescine (Put) synthesis (Pegg, 2006). Degradation of the ODC enzyme occurs when it complexes with a protein known as "antizyme", thereby exposing its C-terminus, which is a region rich in hydrophilic amino acids, such as proline, glutamic acid, serine, and threonine (PEST) (Li and Coffino, 1992, 1993a; 1993b). This PEST sequence is a target for specific protein degradation via the 26S proteasome (Rechsteiner and Rogers, 1996).

Recently, our research group characterized the maize SPMS1 (ZmSPMS1) enzyme, involved in Spm synthesis. *In silico* analysis of ZmSPMS1 identified a potential PEST sequence in the C-terminus. Later, in a functional complementation analysis using a mutant strain of *Saccharomyces cerevisiae* unable to produce Spm (*Δspe4*), we found that only the truncated C-terminal version was able to

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complement the yeast mutant producing Spm (Rodríguez-Kessler et al., 2010). This result suggests that the absence of the PEST sequence generates a functional version of ZmSPMS1 enzyme in yeast.

In this study, we characterized the PEST sequence of the ZmSPMS1 enzyme *in planta*. To this aim, we performed a translational fusion between the β-glucuronidase (*GUS*) reporter gene and 123 bp of the open reading frame of *ZmSPMS1* gene, which includes the PEST sequence. This *GUS*::PEST fusion was assessed by transient expression in onion monolayers and *Arabidopsis thaliana* transgenic lines. Our data show that the PEST sequence of ZmSPMS1 enzyme leads to specific degradation of the GUS reporter protein. This result constitutes the first report of a functional PEST region in plant spermine synthase enzymes.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *A. thaliana* ecotype Col-0 were used. Seeds were sterilized with a 20% (v/v) chlorine solution for 5 min. After three washes with sterile distilled water, seeds were germinated on Murashige and Skoog (MS) 0.5X plates, pH 5.7, containing 0.5% (w/v) sucrose, and 1% (w/v) agar (Murashige and Skoog, 1962). The plates were incubated in a growth chamber with a photoperiod of 16 h light/8 h dark cycle at a temperature of 22 ± 2 °C for seven days.

The onion monolayers were obtained from an *Allium cepa* commercial variety and used for the transient expression experiments as described below.

2.2. Vector construction

The pMDC32-*GUS* construct was generated by PCR amplification of the *GUS* open reading frame. The amplified product was cloned into the pCR8/GW/TOPO entry vector (Invitrogen, Carlsbad, CA, USA) and subcloned into the pMDC32 binary vector (Curtis and Grossniklaus, 2003) by site-specific recombination using Gateway LR Clonase II Enzyme Mix (Invitrogen). The oligonucleotides used were as follows: 5-*GUS* 5′-atgttacgtcctgtagaaaccccaacc-3′ (sense) and 3-*GUS* 5′-ttattgtttgcctccctgctgcgg-3′ (antisense).

The pMDC32-*GUS*::PEST construct was generated by PCR using the *GUS* open reading frame without stop codon and the last 123 bp of *ZmSPMS1* gene containing the nucleotides encoding the PEST sequence. In order to fuse the 3′ end of *GUS* gene to the 5′ end of *ZmSPMS1* PEST region, *Kpn*I restriction sites were introduced in the oligonucleotide sequence (underlined bases) as described: 5-*GUS* 5′-atgttacgtcctgtagaaaccccaacc-3′ (sense) and 3-*GUSKpn*I 5′-gg ggtaccttgtttgcctccctgctg-3′ (antisense) for the *GUS* gene, and 5-Zm PEST*Kpn*I 5′-ggggtaccagagaactggaagcatatgccgct-3′ (sense) and 3-Zm PEST 5′-ctaggaagctgtaagaatggc-3′ (antisense) for the PEST sequence.

After amplification, both PCR products were digested with *Kpn*I enzyme (Invitrogen) generating cohesive ends and then fused using T4 DNA ligase (Invitrogen). The ligated product was cloned into the pCR8/GW/TOPO entry vector and subcloned into the pMDC32 binary vector by site-specific recombination using Gateway LR Clonase II Enzyme Mix (Invitrogen).

2.3. Agrobacterium tumefaciens-mediated transformation

The generation of *A. thaliana* transgenic lines was performed using the *A. tumefaciens* strain GV2260 containing the vector pMDC32-*GUS* or pMDC32-*GUS*::PEST by the "floral dip" method (Zhang et al., 2006). Transformed plants were selected on MS medium supplemented with 50 mg/mL hygromycin B. Seven independent transgenic lines of the pMDC32-*GUS*::PEST construct were obtained, and the T3 generation of the 35S:*GUS* L1 (control) 35S:*GUS*::PEST L1 and L2 lines were used for all the experiments.

Transient expression in onion monolayers was performed using the *A. tumefaciens* strain GV3101 according to the Feng et al. (2008) protocol, with some modifications. The onion monolayers were incubated on MS liquid medium containing the following: *A. tumefaciens* GV3101 transformed with the recombinant vector, 50 mM acetosyringone and 10 mM $MgCl_2$, for 40 min. After that, the onion monolayers were recovered on MS liquid medium supplemented with 0.5% sucrose for 12 h in the dark at a temperature of 22 ± 2 °C.

2.4. RNA isolation and RT-PCR analysis

The RNA isolation of *A. thaliana* seedlings was carried out using Concert reagent (Invitrogen) according to manufacturer's instructions.

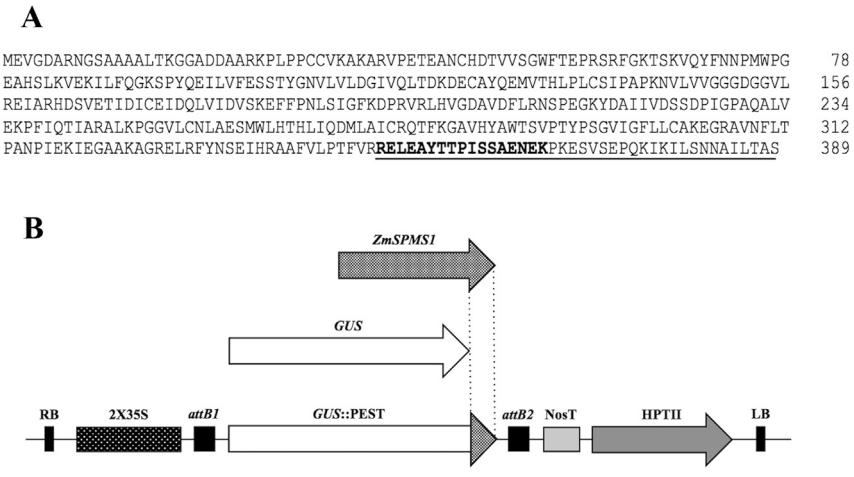

Fig. 1. The pMDC32-GUS::PEST vector. A) ZmSPMS1 amino acid sequence. Underlines show the 40 amino acids of the C-terminal region of the ZmSPMS1 enzyme, and the bold sequence corresponds to the predicted PEST region. B) Schematic representation of *GUS* reporter gene fused to the last 123 bp of the *ZmSPMS1* gene (in gray), which contains the nucleotide sequence that encodes the PEST region. This fusion was introduced by site-specific recombination into the pMDC32 binary vector, which contains the RB right border for T-DNA integration, 2×35S cauliflower mosaic virus 35S promoter, *att*B1 and *att*B2 sites for recombination, *GUS*::PEST fusion, NosT nopaline synthase (nos) terminator region, HPTII hygromycin resistance gene, and LB left border for T-DNA integration.