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Research article

Arabidopsis *Polyamine oxidase-2* uORF is required for downstream translational regulation

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ABSTRACT

In eukaryotic mRNAs, small upstream open reading frames (uORFs) located in the 5′-untranslated region control the translation of the downstream main ORF. Polyamine oxidase (PAO) enzymes catalyze the oxidation of higher polyamines such as spermidine and spermine, and therefore contribute to the maintenance of intracellular polyamine content and to the regulation of physiological processes through their catabolic products. Recently, we reported that the *Arabidopsis thaliana Polyamine Oxidase 2* (*AtPAO2*) is post-transcriptionally regulated by its 5′-UTR region through an uORF. In the present study, we analyzed whether the translation of the uORF is needed for the translational repression of the main ORF, and whether the inactivation of the uORF had an effect on the translational control mediated by polyamines. To this aim, we generated diverse single mutations in the uORF sequence; these mutant 5′-UTRs were fused to the *GUS* reporter gene, and tested in onion monolayer cells and *A. thaliana* transgenic seedlings. Removal of the start codon or introduction of a premature stop codon in the *AtPAO2* uORF sequence abolished the negative regulation of the GUS expression exerted by the wild-type *AtPAO2* uORF. An artificial uORF (32 amino acids in length) generated by the addition of a single nucleotide in *AtPAO2* uORF proved to be less repressive than the wild-type uORF. Thus, our findings suggest that translation of the *AtPAO2* uORF is necessary for the translational repression of the main ORF.

1. Introduction

Polyamines (PAs) such as putrescine (Put), spermidine (Spd), and spermine (Spm) are ubiquitous polycations with wide-ranging functions in diverse cellular processes (Handa and Mattoo, 2010). In plants, Put is produced through the ornithine decarboxylase (ODC, EC 4.1.1.17) or arginine decarboxylase (ADC, EC 4.1.1.19) pathway (Fig. S1). Then, Put is converted to Spd by Spd synthase (SPDS, EC 2.5.1.16), in turn Spd is converted to Spm or thermospermine (T-Spm) by Spm synthase (SPMS, EC 2.5.1.22) and thermospermine synthase (TSPMS, EC 2.5.1.B4), respectively. The formation of Spd, Spm, and T-Spm involves the addition of aminopropyl groups from decarboxylated *S*-adenosylmethionine (dcSAM) that is produced by the *S*-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50) (Fig. S1). PAs exert different roles in fundamental biological processes such as replication, transcription, and translation. In plants, PA homeostasis is critical for a number of physiological functions that include the control in N:C balance, growth and development, and the response to abiotic and biotic stresses (Moschou et al., 2012; Tavladoraki et al., 2012). Therefore, cellular PA content is tightly regulated by biosynthesis, transport, conjugation, and degradation. Polyamines are catabolized by the action of copper-containing diamine oxidases (CuAOs, EC 1.4.3.6) and flavin-containing polyamine oxidases (PAOs, EC 1.5.3.3). Plant CuAOs are localized in the apoplast and peroxisomes, and they participate mainly in the oxidation of Put, cadaverine, and Spd (Planas-Portell et al., 2013; Tavladoraki et al., 2016). The reaction products are 4-aminobutanal that spontaneously cyclizes to Δ^1pyrroline, hydrogen peroxide (H_2O_2), and NH_3 (Fig. S1) (Planas-Portell et al., 2013; Tavladoraki et al., 2016). PAOs catalyze the oxidative deamination of Spd and Spm or their acetylated derivatives (Tavladoraki et al., 2012). Plant PAOs such as maize ZmPAO1, barley HvPAO1, and rice OspAO7 participate in the terminal catabolism of Spd and Spm,

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which takes place in the apoplast, producing 4-aminobutanal and N-(3-aminopropyl)-4-aminobutanal, respectively, along with 1,3-diaminopropane (DAP) and H_2O_2 (Fig. S1) (Federico et al., 1990; Cervelli et al., 2006; Liu et al., 2014). PAOs and CuAOs contribute to the maintenance of PA homeostasis and modulate several PA-linked physiological roles through their catabolic products. For instance, PAO-generated H_2O_2 participates in stomatal movement, pollen tube growth and development, signaling, and programed cell death (Moschou et al., 2008a; Wu et al., 2010; Hou et al., 2013).

The *Arabidopsis thaliana* genome contains five PAO-encoding genes, termed *AtPAO1* to *AtPAO5*. Arabidopsis PAOs exhibit important differences in tissue localization and temporal expression pattern, and in the substrate specificity (Fincato et al., 2012). AtPAO1 and AtPAO5 are cytoplasmic enzymes that convert Spm or T-Spm to Spd in the so-called partial back-conversion pathway (Fig. S1); although with a low efficiency, AtPAO1 is also able to oxidize norspermine (Nor-Spm) through a terminal catabolic pathway (Tavladoraki et al., 2006; Moschou et al., 2008b; Fincato et al., 2011; Kim et al., 2014). AtPAO2, AtPAO3, and AtPAO4 are peroxisomal proteins and participate in a partial back-conversion (Spm to Spd), or in a full back-conversion pathway where Spm is first converted to Spd and then to Put with the concomitant production of H_2O_2 (Fig. S1) (Tavladoraki et al., 2006; Kamada-Nobusada et al., 2008; Moschou et al., 2008b; Takahashi et al., 2010; Fincato et al., 2011; Kim et al., 2014).

In both mammals and plants, polyamine metabolism is controlled by complex regulatory mechanisms, one of which is translational regulation. Upstream open reading frames (uORFs) are located in the 5′-untranslated region (5′-UTR) and modulate the translation of the main ORF (mORF). These regulatory elements have been identified in genes encoding transcription factors, protein kinases, enzymes, receptors, and proteins involved in translation and degradation (Calvo et al., 2009; Jorgensen and Dorantes-Acosta, 2012). The mORF translation regulation can be dependent or independent of the uORF amino acid sequence (Lohmer et al., 1993; Hanfrey et al., 2005; Ivanov et al., 2008). For instance, the nascent uORF peptide can stall ribosomes thereby inhibiting the translation of the downstream ORF. Accordingly, some uORF peptides characterized so far cause ribosomal arrest during the elongation or the termination step by forming peptidyl-tRNA complexes (Raney et al., 2002), or once the uORF peptide is translated (Combier et al., 2008). However, the cell has evolved several mechanisms to counteract the effect of uORFs, which include leaky scanning, shunting, and reinitiation (Rahmani et al., 2009; Roy et al., 2010). In leaky scanning, the ribosome ignores the first 5′-proximal ATG codon and the translation begins at a downstream ATG (Haimov et al., 2015). Translation reinitiation is a process in which, after translation termination the 40S ribosome reacquires some initiation factors such as eIF2 ternary complex and eIF3h, resumes scanning and initiates the translation at downstream ORF (Roy et al., 2010; von Arnim et al., 2014). Both ribosomal reinitiation and leaky scanning have been reported for plant mRNAs containing uORFs, for instance, the AtbZip11 transcription factor (Roy et al., 2010). During ribosome shunting, the 40S subunit bypasses a part of the 5′-UTR; however, this mechanism has not been demonstrated in plants (von Arnim et al., 2014).

In plants, uORFs are common in polyamine metabolic genes, even though the function and importance of uORF-encoded amino acid sequences has been proved only for *AtSAMDC1* and carnation *ADC* genes (Chang et al., 2000; Hanfrey et al., 2005; von Arnim et al., 2014). Recently, we reported the presence of conserved uORFs in *PAO2, PAO3*, and *PAO4* transcripts from several plants (Guerrero-González et al., 2014). Furthermore, we characterized the uORF of the *A. thaliana AtPAO2*, and demonstrated that the *AtPAO2* gene is post-transcriptionally regulated through its 5′-UTR in a PA-dependent way. In this study, we analyzed whether the translation of the uORF is needed for the translational repression of the main ORF, through the characterization of diverse single mutations in uORF sequence. Mutation of the ATG start codon or the introduction of a premature stop codon in the uORF alleviated the negative effect of the *AtPAO2* WT-uORF on the mORF translation. In addition, we analyzed the effect of a new uORF of 32-amino acids in length that was generated by a frameshift mutation in the *AtPAO2* uORF sequence. This artificial uORF negatively regulated GUS expression levels, but independently of an exogenous application of Put and Spd. These findings indicate that the peptide derived from the *AtPAO2* uORF is necessary for translational regulation of the *AtPAO2* mORF.

2. Materials and methods

2.1. Plasmid construction

The plasmid 35S-UTR::GUS previously described (Guerrero-González et al., 2014) was used as template. This plasmid contains the *AtPAO2* 5′-UTR sequence, which is fused upstream to the *GUS* (*β-glucoronidase*) reporter gene, and contains the *AtPAO2* uORF coding sequence. In order to eliminate the ATG start codon of the uORF, the adenine nucleotide was changed to thymine (A→T) giving rise to TTG codon, a 280 bp fragment from the *AtPAO2* 5′-UTR was amplified employing the oligonucleotide UTRPAO2-Fw 5′-CCAAAGCCTAAAAAATCCGACC-3′ and the mutagenic oligonucleotide uORTTG-Rv 5′-TTCCAAACAAATTCAAGCGATTTTCTTCTTC-3′ (the changed nucleotide is underlined). Additionally, a 2757 bp fragment that includes 189 bp of the *AtPAO2* 5′-UTR plus the *GUS* gene was amplified using the mutagenic oligonucleotide uORTTG-Fw 5′-AAGAAAATCGCTTGAATTTGTTTGGAA-3′ (the changed nucleotide is underlined) and the oligonucleotide GUS-stop 5′-TTATTGTTTGCCTCCCTGCTGCGG-3′. Both fragments were overlapped and extended using the oligonucleotides UTRPAO2-Fw and GUS-stop with Phusion High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA). The thermal cycling conditions consisted of 30 s at 98 °C, followed by 30 cycles of 10 s at 98 °C (denature), 30 s at 64 °C (anneal) and 1 min 45 s at 72 °C (extension), with a 5 min at 72 °C for final extension. The PCR fragment was introduced into the pCR®8/GW/TOPO vector (Invitrogen, Carlsbad, CA, USA). Then, the clone was recombined into the binary vector pMDC32 by the Gateway LR Clonase Enzyme mix (Invitrogen, Carlsbad, CA, USA) downstream from the constitutive cauliflower mosaic virus (CaMV) *35S* promoter, to generate the TTG plasmid.

Using the above-mentioned procedure, the TGA plasmid was generated in which the third codon of the uORF was replaced by a stop codon (TTG→TGA). The UTRPAO2-Fw and the mutagenic uORTGA-Rv 5′-AAAAAATCAGGCTTCAAAATCAATTCATGC-3′ (the changed nucleotides are underlined) oligonucleotides were used to amplify a 280 pb fragment, while the oligonucleotides uORTGA-Fw 5′-GCATGAATTGATTTTGAAGCCTGATTTTTT-3′ (the changed nucleotides are underlined) and the GUS-stop were used to amplify a 2757 pb fragment. To generate the New Peptide (NP) plasmid, an extra nucleotide was introduced in the uORF coding sequence, between the +7 and + 8 position, by overlapping a 280 pb fragment, that was amplified using the UTRPAO2-Fw oligonucleotide and the mutagenic oligonucleotide npuORF-Rv 5′-TTCCAAACA-CAATTCATGCGATTTTC-3′ (the inserted nucleotide is underlined) with a 2757 pb fragment, which was amplified using the npuORF-Fw 5′-AAAATCGCATGAATTGTGTTTGGAAG-3′ and the GUS-stop oligonucleotides. The sequences of all plasmids carrying the mutations were confirmed by sequencing both DNA strands.