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Molecular details of the Raptor-binding motif on *Arabidopsis* S6 kinase

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ABSTRACT

A putative raptor-binding fragment was identified from *Arabidopsis* S6 kinase 1 (AtS6K1) N-terminal domain in our previous study. Here, we report a further characterization of this fragment, which identified a 12-amino acid core element absolutely required for the interaction. Although the amino acid sequence of the element *per se* had no significant homology with the canonical consensus of the TOS (TOR-signaling) motif found in the mammalian TOR (target of rapamycin) kinase substrates, its overall sequence composition is similar to that of the TOS motif in that the acidic and non-polar amino acids residues are arranged in alternating fashion and having one or two of the bulky hydrophobic amino acid (F) buried in the interior. Substitution of this bulky residue completely abolished the binding of the fragment to AtRaptor1, as in the case of the mammalian TOS motif. Taken together with its position relative to the catalytic domain of the kinase, which also shows a resemblance with the TOS motif, these results appear to suggest that this core binding element in the N-terminus of AtS6K1 represents a plant version of the TOS motif.

1. Introduction

Recent progress in researches on plant TOR (*t*arget *o*f *r*apamycin) kinase signaling revealed its remarkable multi-tasking capability in the control of cellular growth metabolism, now expanded in the area including the sugar-sensing and the response signaling [1–3], autophage [4,5], and in the leaf development [6], in addition to its classical role of modulating cell growth and proliferation under normal and stress conditions [4,7–9]. Although many details of the signal components in plant TOR pathway differ from those of the mammalian cell or yeast, the basic skeleton of plant TOR pathway is also highly conserved. For example, plant TOR kinase also needs to bind with Raptor (*r*egulatory *a*ssociated *p*rotein of m*TOR*) in order to recruit its substrates such as the ribosomal S6 kinase (S6K) [8,10]. In animals and yeasts, it has been known that the interaction between Raptor and the TOR substrates is dependent upon the TOS (TOR-signaling) motif, which is a specific sequence motif conserved in all TOR substrate proteins [11–13]. However, such conserved sequence element has not been found in the substrates for plant TOR kinase known to date, such as the *Arabidopsis* ribosomal S6 kinase 1 (AtS6K1).

In our previous study, we identified a Raptor-binding region in the N-terminus of AtS6K1. This N-terminal 44-amino acids fragment of AtS6K1 was confirmed to be sufficient for binding with the AtRaptor1 in the yeast two-hybrid analysis, and its expression in the *Arabidopsis* protoplasts resulted a significant reduction in the rDNA transcription, suggesting that it could be potentially used as a plant TOR-signaling inhibitor [14]. As we have pledged in the previous study to continue to further analyze the fragment and obtain a better insight into its molecular and biochemical characteristics in mediating the interaction with the AtRaptor1, here we present additional details about this fragment to reveal its core structure and the information obtained from mutagenesis of selected amino acid residues within the region. Our tentative conclusion with this study is that it is highly likely that this region contains a plant-version of the TOS motif through which the molecular interaction between plant Raptor and its substrates is maintained.

2. Materials and methods

2.1. Yeast two-hybrid assay

Yeast strain and methods used in the yeast two-hybrid assay

Abbreviations: Raptor, Regulatory associated protein of mTOR; S6K1, ribosomal S6 kinase 1; TOR, target of rapamycin.

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were essentially the same as in our previous report [14]. The various portions of AtRaptor1 cDNA were subcloned into pGBKT7 as baits, while AtS6K1 partial cDNAs were subcloned into pGADT7 as preys, respectively (Clonetech Inc.). Primers used were shown in Supplementary Table S1.

2.2. Protoplast transfection

The partial cDNAs and mutagenized versions of AtS6K1 were cloned into the p326GFP vector. Primers used were shown in Supplementary Table S1. Each plasmid was transfected into *Arabidopsis* (ecotype Columbia) protoplasts for transient expression [14]. Transfected protoplasts were used for total RNA isolation.

2.3. Real-time RT-PCR

Methods described previously [14] were followed and primers used in this study were shown in Supplementary Table S1.

3. Results and discussions

3.1. A putative plant TOS motif was identified through N-terminal deletion of AtS6K1

In our previous study, we generated serial deletions in the N-terminus of the *Arabidopsis* ribosomal S6 kinase 1 (AtS6K1) and tested each of these fragments' binding ability to the *Arabidopsis* Raptor (AtRaptor1) to identify any sequence motif similar to the TOR-Signaling (TOS) motif found in mammalian TOR-substrates [12,14]. Although we were not able to locate such motif in the study, we identified a region of 44-amino acids that were responsible for the interaction with the full-length AtRaptor1 protein fragment. To further fine-tune this analysis and define more precisely the region of the AtS6K1 that is directly involved in the interaction with AtRaptor1, we continued to narrow down this fragment in this study using the same yeast two-hybrid analysis.

First, we wanted to be able to draw a more concrete conclusion about the regions in AtRaptor1 that are involved in the interaction with AtS6K1. In the previous study, none of the partial fragments generated by serial deletion of the AtRaptor1 protein could interact with AtS6K1. Although partial fragment of the AtRaptor1 protein, such as the C-terminal WD40 repeats, was sufficient for the interaction with a few other proteins that were also tested for in the same study, only the full-length AtRaptor1 was able to interact with the AtS6K1. To test if both N- and C-terminal regions of the AtRaptor1 are required in the case of interaction with the AtS6K1, we generated a chimeric construct in which either the N-terminal RNC domain of AtRaptor1 was linked to the C-terminal WD40 repeats via green fluorescence protein (GFP) inserted in between as a stuffer. However, this construct did not show any positive interaction with either the full-length AtS6K1 or the N-terminal 44 amino acid fragment tested by the yeast two-hybrid analysis (data not shown). According to this result, therefore, we came to a tentative conclusion about the nature of the interaction between AtRaptor1 and AtS6K1 as that the overall topology of the Raptor may be important in maintaining the interaction.

Next, we further scrutinized the N-terminal 44 amino acid fragment of AtS6K1 (Nt12-12 in Fig. 1) to see if it was the minimal fragment absolutely required for the interaction with AtRaptor1. The TOS motif conserved in the mTOR substrates such as mammalian p70 S6 kinase (S6K1) and 4E-BP consists of about 12 amino acids [12,13]. Therefore, we generated additional sets of deletions starting with this 44-amino acid fragment and assayed each fragment's binding affinity for AtRaptor1 by yeast two-hybrid analyses. The result showed that a 12-amino acid fragment further down towards the C-terminus of the original fragment (c26-12 in Fig. 1) could interact with the AtRaptor1 as strongly as the original 44-amino acid fragment. By outright sequence comparison, this fragment (5′-DDVELEFSDVFG-3′) does not show any significant homology with the consensus TOS motif such as 5′-MAGVF-DIDLDQP-3′ found in human S6K1 [12]. However, its relative position in the overall architecture of the kinase as well as its amino acid composition are somewhat similar to those of the TOS motif. In the case of mammalian S6K1, for example, the TOS motif starts with the first methionine codon of the protein and this marks about 70 amino acids (aa) away from the start of the catalytic domain. On the other hand, this 12-amino acid fragment of the AtS6K1 is positioned at 28 aa after the start codon, and it is 112 aa away from the start of the catalytic domain (Fig. 2). In effect, this difference in the relative position of these two elements reflects the intrinsic difference in the structure of N-terminal domains of these two kinases. The N-terminus of AtS6K1 is twice as long as that of the mammalian S6K1. This extended N-terminus of the plant S6K1, including AtS6K1, makes up for its relatively short C-terminus. Unlike the mammalian S6K1 that carries a C-terminal domain that is relatively extended (about 170aa long) and highly phosphorylated, the C-terminus of AtS6K1 is short (only about 70aa long) and has fewer phosphorylation sites than that of the mammalian S6K1 [15]. We have demonstrated in our earlier study [15] that, unlike the mammalian S6K1, the N-terminal domain of AtS6K1 is phosphorylated, which supports its compensatory role for the short and under-phosphorylated C-terminus. Therefore, it is understandable that a sequence element equivalent to the mammalian TOS motif is present in an internal region of the N-terminus of AtS6K1.

3.2. Binding of AtS6K1 with AtRaptor1 can be abolished by a point mutation

As stated above, this putative “TOS motif” of AtS6K1 bears no sequence homology with the canonical mammalian TOS motif sequence, but the amino acids composition of the element is comparable with that of the TOS motif in that acidic residues alternate with non-polar amino acids. In the case of the human S6K1, it has been shown that several point mutations introduced in the TOS motif region resulted in almost complete inactivation of the kinase activity and the phosphorylation in the C-terminus of the kinase, which indicates failure of the binding to Raptor [12]. Most notable among them was a mutant in which the amino acid phenylalanine was converted to alanine (F5A) that resulted in a complete inactivation and the lack of phosphorylation of the kinase [12]. We tried similar point mutations in several residues present in the putative TOS motif of AtS6K1 and tested their impacts on the binding affinity of AtS6K1 to the AtRaptor1 by yeast two-hybrid analysis as shown in Fig. 3. All three mutants we tested, G40A, F35S, and F39S, significantly affected the binding of the fragment to AtRaptor1. Besides, all these mutations were introduced within the context of the original 44-amino acid fragment, rather than the narrowed-down 12-amino acid context, and still abolished the interaction almost completely, which indicates the significance of these residues for the structural integrity of the motif required for the binding between AtS6K1 and AtRaptor1. Whether this 12-amino acid element indeed plays a role of TOS motif in plant TOR signaling or not could only be determined after identifying more plant TOR substrates proteins and examining their amino acid sequences and domain architectures more rigorously.

3.3. The point mutation in Raptor-binding motif also affect plant TOR signaling

In our previous study, we showed that the N-terminal 44-aa

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