FUNCTIONAL CHARACTERIZATION OF RNA POLYMERASE III SUBUNIT RPC4 IN *ARABIDOPSIS THALIANA*

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SUMMARY

In Eukaryote, RNA polymerase III is dedicated to the transcription of tRNA molecules, 5S rRNA and other small RNA. The gene encoding protein RPC53, one of the subunits in RNA polymerase III complex, is shown to be an essential gene which specifically regulates tRNA gene transcription *in vivo* in *Saccharomyces cerevisiae*. However, the function of this protein in plant system is not known yet. A BLAST search of the database using the amino acid sequence of the 42-kDa Rpc53p *S. cerevisiae* as query revealed one striking match with a 30-kDa protein encoded RNA polymerase III subunit RPC4 in *Arabidopsis*. *Arabidopsis rpc4* gene expression was analyzed in different tissues. RPC4 protein is widely expressed in *Arabidopsis* and green fluorescent protein-RPC4 fusion protein localizes specifically to the cell nucleus, forms two different patterns. *Arabidopsis* lines harboring T-DNA insertions in the coding sequence of *rpc4* were characterized by genotyping. Characterization of mutant phenotype showed that *rpc4* loss of function mutant displayed growth defects, observed in smaller leaf size, delayed flowering and delayed senescence. These phenotypes illustrated an essential function of RPC4 in regulation of growth and development. This finding supported the first characterization of RPC4 protein in *Arabidopsis* development, opened up an important possible mechanism regarding to regulation of tRNA transcription in plant development.

Keywords: *Arabidopsis*, Genotyping, RNA polymerase III subunit 4 RPC4, Rpc53p *Saccharomyces cerevisiae*, T-DNA insertions.

INTRODUCTION

Transcription, that is, RNA synthesis on a DNA template, is performed by DNA-dependent RNA polymerases (Pols). Eukaryotic cells contain three different Polymerase I, II, and III, which differ in subunit composition. The difference probably reflects their evolutionary specialization in transcription of different genes and suggests their independent regulation.RNA polymerase I (RNA Pol I) synthesizes the large ribosomal RNAs (rRNA), RNA polymerase II (RNA Pol II) produces mRNAs and many noncoding RNAs, RNA polymerase III (RNA Pol III) transcribes genes encoding short untranslated RNAs such as transfer RNAs (tRNAs),5S ribosomal RNA (rRNA), U6 snRNA, 7SK RNA and 7SL RNA; Alu repeats; some viral genes; and genes for small stable untranslated RNAs. These genes are essential and involved in fundamental processes like protein biogenesis; hence RNAP III activity needs to be tightly regulated (White *et al.*, 2011; Werner *et al.*, 2009; Flores *et al.*, 1999). The subunit composition of yeast and human Pol III was well studied in the previous reports. In *Saccharomyces cerevisiae*, RPC53 is shown to be an essential gene encoding the C53 subunit specifically associated with RNA polymerase C.Temperature-sensitive *rpc53* mutants showed a rapid inhibition of tRNA synthesis after transfer to the restrictive temperature. The C53 subunit has no paralogue in the two other nuclear RNA polymerases, and is therefore one of the five subunits specific to Pol III. A functional C53 protein is required for yeast cell viability and that inactivation of C53 temperature-sensitive mutants rapidly leads to an inhibition of tRNA gene transcription *in vivo* (Mann *et al.*, 1992). In plants, RPC53 has not been studied so far. Here we report the identification of a nuclear protein with striking sequence similarity to RPC53 that is encoded in *Arabidopsis*, and we examine mutant plants lacking this protein in comparison to control plants.

MATERIALS AND METHODS

Plant material and growth conditions: All plants were grown in an environmentally controlled growth room at 22°C with a photoperiod of 16-h day light.For phenotypic assays, seeds were cold-treated at 4°C for 3 days, sown directly in the soil transferred to white light intensity (normal light intensity) (85μmol.m^{-2}.s^{-1}). To characterize *rpc4* mutant phenotype, plant also were grown under low light intensity (25μmol.m^{-2}.s^{-1}).

RT-PCR gene expression and small RNA analysis: After 2 weeks of growth, for RNA extraction, leaves, roots, flowers from mature plants were sampled. Total RNA was prepared by manual TRIZOL method and cDNA was synthesized from 2μg of the total RNA with PrimeScript Reverse Transcriptase (Takara Bio) using an oligo(dT) primer. RT-PCR analysis of RPC4 (AT5G09380) and RPC4 homologous gene (At4g25180) expressions were performed using specific primer pairs. ACTIN8 was used as an internal control gene.The RNA was then reverse transcribed. Combine the following in a microfugetube:oligo T 1 μl, RNA 2μl, and distilled istilled water 2μl; heat mixture at 65°C for 5 min. For quantification of cDNA, set up the following components in a 0.5ml PCR tube: cDNA product 5μl, $MgCl_2$ 2.4μl, dNTP 1μl, inhibitor 1μl, Rtase 1μl, and distilled water 5.6μl. The following conditions were used: 42°C for 1 hour, then 65°C for 5 min and keep at 15°C.

PCR-based genotyping of plant lines To distinguish among plants that are wild type, heterozygous for T-DNA insertions, or homozygous for T-DNA insertions, genomic DNA was isolated from leaves The genomic DNA was used for PCR analysis with Taq DNA polymerase (Amplicon) and primers specific for T-DNA *rpc4*.

RESULTS AND DISCUSSION

Arabidopsis genome RPC4 encodes a protein with striking similarity to RPC53 in yeast

RPC53 is shown to be an essential gene encoding the C53 subunit specifically associated with yeast RNA polymerase

C (III). In *S. cerevisiae*, C37–C53 was identified as a key role of the complex in the recognition of the terminator elements, *rpc37*, *rpc53* and, perhaps indirectly, Rpc11 contribute to switch Pol III from elongation to termination (Emilie *et al.*, 2006). In human, it has recently been shownthat the predicted BN51 protein has a significant homology to the 53 kD subunit (C53) of RNA polymerase C(III) from *S. cerevisiae* (Michael *et al.*, 1999). In plants, RPC53 has not been studied so far. In view of the conservation of RPC4 in organisms as different as yeast and mammals, and the study of RNA polymerase III in yeast was well known. Therefore, we wondered whether there is also a RPC4 protein related to yeast RPC53. We have performed a BLAST search of the *Arabidopsis thaliana* database using the amino acid sequence of the 42-kDa *RPC53 S. Cerevisiae* as query.The search revealed one striking match with a 30-kDa protein encoded in the *Arabidopsis* genome (AGI locus At5g09380) termed in the following RPC4,which contains C-domain belongs to RNA polymerase-*Rpc4* superfamily (Figure 1). The alignmental so reveals that the plant RPC4 sequences display significant similarity to typical RPC53 proteins, primarily in the C-terminal. RPC4 shows 41% identity and 68% similarity to RPC53 of yeast (*S. cerevisiae*). In addition, RNA polymerase III subunit 4 was encoded by two genes At5g09380 and At4g25180 in *Arabidopsis*, sharing 32% identity (data not shown).

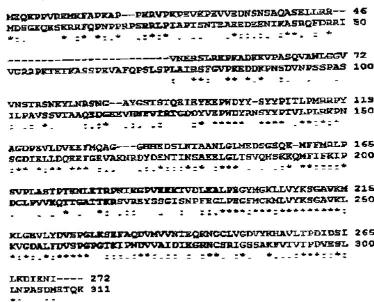

Figure 1. Alignment of protein sequence of RPC4 protein in *Arabidopsis* to that of C53 subunit of yeast RNA polymerase III. *Upper sequence*, the C53 protein of *S. cerevisiae*; *lower sequence*, the RPC4 sequence of *Arabidopsis*. The protein sequences were aligned by multiple sequence alignment (ClustalW).

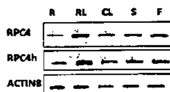

Figure 2. *Arabidopsis* RPC4 is ubiquitously expressed. RT-PCR analysis of total RNA isolated from the roots (R), cauline leaves, rosettle leaves, flowers and stem (from 4-week-old plants). PCR was performed with primers specific for RPC4 and RPC4 homolog (RPC4h) (top and second, respectively) and ACTIN8 (loading control) (bottom). R: roots, CL: cauline leaves, RL: rosette leaves, S: stem, F: flowers

Arabidopsis RPC4 is ubiquitously expressed, and a green fluorescent protein–RPC4 fusion localizes to the cel nucleus

To examine the expression of RPC4 proteins, RNA isolated from different tissues and reverse transcription was used fo detection of the RPC4 transcripts by PCR. Using RPC4 and RPC4 homolog specific primers, amplification products o the expected size (819 and 936 for RPC4 and RPC4 homolog, respectively) were obtained from all RNA samples, whict were derived from the roots, cauline leaves, rosettle leaves, flowers and stem (from 4 week-old plants). Amplification o ACTIN8 served as loading control. The reverse-transcribed polymerase chain reaction experiment indicated that RPC4 i ubiquitously expressed in *Arabidopsis* and that the expression levels differ. For instance, there is a lower amount of the *rpc4* transcript in roots and cauline leaves while the expression of RPC4 is higher in rosettle leaves and flower. Th same patterns were observed in case of RPC4 homolog protein (Figure 2). We also looked for available databases i Genevestigator. Data shows that RPC4 is expressed during every development stages in *Arabidopsis* but highl expressed in vegetative and senescence stages. However, the database for RPC4 homolog protein is not available unt now (Genevestigator)

Since RPC4 typically is considered a nuclear protein, we have examined the sub-cellular localization of *Arabidops* RPC4. We have constructed plasmids suitable for the expression of green fluorescent protein (GFP) fusion proteins i plant protoplasts. In transient transformation assays performed with *Arabidopsis* cell protoplasts, the expression of th GFP fusion proteins was driven by the CsVMV promoter. Transformed protoplasts were analyzed by confocal lase

scanning microscopy. Nuclear RPC4 protein formed two different patterns, both dispersed form and speckle form in nucleus. The cytoplasm background GFP was used as in the negative control (Figure 3).

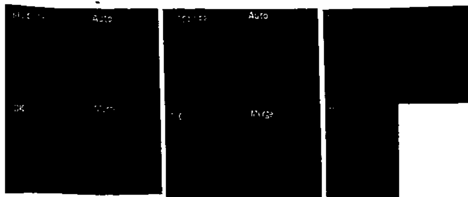

Figure 3. Nuclear localization of RPC4 and RPC4 homolog (At4g25180-GFP) using GFP-fusion construct transient expressed in *Arabidopsis* protoplast, analyzed by confocal laser scanning microscopy.

T-DNA insertion knockout mutants selection

The transcribed region of the *Arabidopsis* genes (AGI locus At5g09380) encoding RPC4 and RPC4 homolog (AGI locus At4g25180) consist of 10 and 9 exons, respectively (Figure 4A). To elucidate the physiological functions of RPC4 *in planta*, T-DNA insertion mutant *rpc4-1 (SALK_002157)*, *rpc4-2(SALK_098495)* and *rpc4h-1 (SALK_125873) and rpc4h-2 (SALK_025232)* from the SALK collection were analyzed.The insertion sites were confirmed by PCR analysis of genomic DNA, in combination with DNA sequencing of PCR fragments spanning the left borders of the T-DNA. These analyses revealed that we were able to isolate plants homozygous for the T-DNA insertions of RPC4, termed *rpc4-1*, *rpc4-2* and homologous RPC4, termed *rpc4h-1*, *rpc4h-2* (Figure 4B). RNA isolated from *rpc4-1*, *rpc4h-1* was examined by reverse-transcribed PCR (RT-PCR). Based on RT-PCR analysis of RNA isolated from *rpc4-1 and rpc4h-1* homozygous plants, no transcript of the RPC4 and RPC4h gene was detected, whereas the corresponding DNA fragment of 819bp and 936bp, respectively are readily amplified by RT-PCR from wild type RNA (Figure 4C).

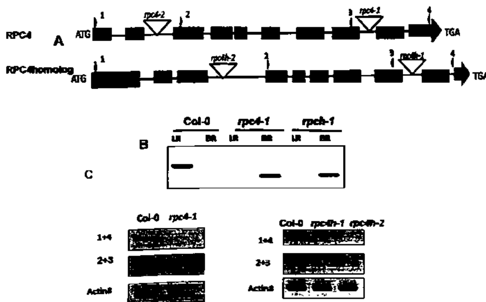

Figure 4. Characterization of *Arabidopsis* RPC4 and homologous RPC4 T-DNA insertion lines(A) Schematic representation of the RPC4 gene (top) and RPC4 homolog (RPC4h) (bottom). Boxes represent exons, while lines indicate introns (not drawn to scale). (B) Genotyping T-DNA insertion mutants: *rpc4* and *rpc4 homolog (rpc4h)* homozygous selection LR: LP+ RP. BR: BP+RP. LP, RP: Left, Right genomic primer. BP - T-DNA border primer LB - the left T-DNA border primer. (C) RNA gel blot analysis of *rpc4* and *rpc4 homolog* mutants. *rpc4h-1: RPC4 homolog -1, rpc4h-2: RPC4 homolog -2.*

Phenotypic analysis of *rpc4* mutants

Since function of RPC4 in *Arabidopsis* is still unclear. To unravel the physiological functions of RPC4, we grew wild-type and *rpc4-1*, *rpc4h-1* plants under different light conditions in a growth chamber. Remarkably, *rpc4-1*, *rpc4-2* mutants displayed growth defects observed in smaller leaf size in two weeks grown plant whereas *rpc4h-1* homolog was indistinguishable from wild type under normal light intensity (Figure 5A).

At the flowering stage, the *rpc4-1* mutant reached the wild type leaves size, slightly longer, delayed flowering and delayed senescence (Figure 5B). In yeast, RNA polymerase III was well studied to be regulated through target of rapamycin (TOR) pathway, so that they can control the systemic growth by regulating transcription of tRNA and 5S rRNA. TOR integrates various signals to regulate cell growth. Four major inputs have been implicated in TOR signaling: growth factors, nutrients, energy, and stress cell growth (the accumulation of cell biomass) depends on a high rate of

protein synthesis and consequently requires a high level of cellular energy (Wullschleger *et al.*, 2006). Therefore, We decided to test mutant phenotype under low light intensity, means low energy source for plant. The results showed that low light intensity resulted in significant phenotypes in *rpc4 homolog* K.O mutant. These mutants displayed longer petioles whereas *rpc4-1* showed no difference in petiole length but delayed flowering (Figure 5C). This result revealed the important function of RPC4 in regulation of cell growth and development. Interestingly, the different phenotype of *rpc4* and *rpc4h* mutant in different condition illustrated the possibly differential cross regulation between RPC4 and RPC4 homolog protein in specific environmental conditions.

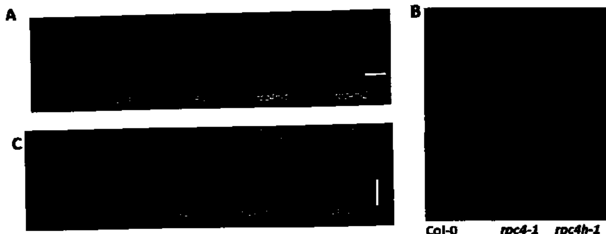

Figure 5. Phenotypic analysis of RNA polymerase subunit RPC4 loss of function mutants, Wild type Col-0, and *rpc4h-1* are included for comparison. All plants were grown under long day conditions. (A) *rpc4-1* mutants display similar growth defects in 2 weeks grown plants under middle light condition. Scale bar=5mm. (B) Mutant plants in vegetative stage. *rpc4-1* showed delayed flowering and delayed senescence. Scale bar = 10mm. (C)Mutants phenotype under low light condition.

CONCLUSION

We have identified a nuclear 272 amino acid residue RPC4 protein encoded in the *Arabidopsis* genome. In *Arabidopsis*, RPC4 is encoded by two homologous genes At5G09380 and At4g25180. Two independent *Arabidopsis* lines homozygous for T-DNA insertions (*rpc4-1* and *rpc4-2*) that disrupt the coding sequence of RPC4; and two independent lines disrupts the coding sequence of At4g25180 were characterized. Accordingly, no wild-type *rpc4* and *rpc4* homolog transcript were detected in the mutant lines. RPC4 revealed an important function in regulation of cell growth and development, which is demonstrated by analyzing knock out mutant. Remarkably, *rpc4-1*, *rpc4-2* mutants displayed growth defects observed in smaller leaf size in two weeks grown plant whereas *rpc4h-1* homolog was indistinguishable from wild type under normal light intensity. At the flowering stage, the *rpc4-1* mutant reached the wild type leaves size, slightly longer, delayed flowering and delayed senescence. This finding supported the first characterization of RPC4 protein in *Arabidopsis* development, opened up an important possible mechanism regarding to regulation of tRNA transcription In plant development.

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NGHIÊN CỨU ĐẶC ĐIỂM CỦA RNA POLYMERASE TIỂU ĐƠN VỊ RPC4 Ở *ARABIDOPSIS THALIANA*

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TÓM TẮT

Ở sinh vật nhân chuẩn, RNA polymerase đóng vai trò quan trọng trong quá trình phiên mã tạo ra các phân tử tRNA, 5S rRNA và các RNA nhỏ khác. Protein RPC53, một trong những tiểu đơn vị của hệ thống phức hợp RNA polymerase III, được nghiên cứu rất nhiều như một thành phần có vai trò cần thiết cho quá trình điều hòa phiên mã tRNA ở *S. cerevisiae*. Tuy nhiên, chức năng của protein này ở hệ thống thực vật vẫn chưa được biết đến. Do đó, sử dụng phương pháp so sánh trình tự BLAST đối với protein RPC53 ở *S. cerevisiae*, chúng tôi đã tìm ra được một protein có trình tự tương đồng rất cao ở *Arabidopsis*, RNA polymerase III tiểu đơn vị RPC4. Phân tích biểu hiện gen mã hóa cho protein RPC4 cho thấy, protein này phân bố ở các cơ quan với mức độ biểu hiện khác nhau. Protein RPC4 phân bố chủ yếu ở trong nhân tế bào, hình thành hai dạng phân bố khác nhau. Phân tích đặc điểm của dạng đột biến mất gen *rpc4-1 và rpc4-2* cho thấy chúng biểu hiện sinh trưởng kém so với dạng hoang dại thể hiện ở kích thước lá nhỏ hơn, chậm ra hoa và chậm già hóa hơn. Những đặc điểm này chứng tỏ vai trò cần thiết của RPC4 trong sinh trưởng và phát triển của *Arabidopsis*. Đây được coi là nghiên cứu đầu tiên về đặc điểm chức năng của protein RPC4, tiểu đơn vị của RNA polymerase III ở *Arabidopsis*, mở ra một cơ chế điều hòa mới, liên quan tới điều hòa quá trình phiên mã tRNA trong sự phát triển của thực vật.

Từ khóa: *Arabidopsis*, Kiểu gen, RNA polymerase III subunit 4 RPC4, Rpc53p *Saccharomyces cerevisiae*, T-DNA insertions.

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