Mutations in the Arabidopsis *Lst8* and *Raptor* genes encoding partners of the TOR complex, or inhibition of TOR activity decrease abscisic acid (ABA) synthesis

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ABSTRACT

The Target of Rapamycin (TOR) kinase regulates essential processes in plant growth and development by modulation of metabolism and translation in response to environmental signals. In this study, we show that abscisic acid (ABA) metabolism is also regulated by the TOR kinase. Indeed ABA hormone level strongly decreases in *Lst8-1* and *Raptor3g* mutant lines as well as in wild-type (WT) Arabidopsis plants treated with AZD-8055, a TOR inhibitor. However the growth and germination of these lines are more sensitive to exogenous ABA. The diminished ABA hormone accumulation is correlated with lower transcript levels of *ZEP*, *NCED3* and *AAO3* biosynthetic enzymes, and higher transcript amount of the *CYP707A2* gene encoding a key-enzyme in abscisic acid catabolism. These results suggest that the TOR signaling pathway is implicated in the regulation of ABA accumulation in Arabidopsis.

1. Introduction

Basic cellular functions of living organisms are regulated by a complex network of biochemical processes and signaling pathways responsible for the regulation of cellular metabolism in response to external signals. The Target of Rapamycin (TOR) pathway is present in all eukaryotic organisms and plays a key role in the regulation of various cellular processes like autophagy, translation, ribosome biogenesis, and metabolic adaptation in response to nutrients, growth factors and energy conditions [1].

The TOR protein kinase is a large protein (~250 kD) which belongs to the phosphatidylinositol kinase-related kinase (PIKK) family [1–3]. In yeast and animals, TOR associates in two complexes TORC1 and TORC2 with different protein partners, namely LST8 and RAPTOR/KOG1 for TORC1, and LST8, RICTOR/AVO3 and SIN1/AVO1 for TORC2 [3]. TORC1 is sensitive to rapamycin and participates in the responses to favorable growth conditions by promoting energy-consuming processes like cell division, translation, ribosome biosynthesis and anabolic metabolism and repressing recycling mechanisms like autophagy [1–3].

In Arabidopsis, a single *TOR* gene and some of the TOR complex 1 (TORC1) partners have been identified including two homologs of the LST8 protein (*Lst8-1* and *Lst8-2* genes) and two genes encoding RAPTOR proteins (*Raptor3g* and *Raptor5g*) [4–8]. Conversely there is no evidence so far for a TORC2 complex in plants [4]. Inactivation of the TOR gene is embryonic lethal [6] but homozygous mutants affected in *Lst8-1* or *Raptor3g* paralogs, which are the most expressed ones, are viable [7,8]. Recently TOR ATP-competitive inhibitors (called asTORis for active-site TOR inhibitors) have been shown to inhibit whole-plant growth in dose-dependent manner [9] indicating that the TOR pathway plays an important role in plant growth.

We previously observed a positive correlation between sensitivity to the plant hormone ABA and TOR expression in Arabidopsis [10]. Despite the growing interest in the plant TOR kinase, this link between ABA and the TOR signaling pathway has received hitherto little attention [4,5]. Furthermore, although it is generally believed that TOR is only active in favorable external conditions, more and

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more studies suggest that the activity of the TOR complex is also needed for stress adaptation [11,12]. ABA plays also an essential role as regulator in many cellular processes including germination, seed development and environmental stress responses [13–15]. The first committed steps of ABA biosynthesis take place in plastids and are catalyzed by zeaxanthin epoxidase (ZEP) and the 9-cis-epoxycarotenoid dioxygenase (NCED) which produces xanthoxin and is thought to be the main rate-limiting reaction [14]. Xanthoxin then moves to the cytoplasm where it is converted to ABA-aldehyde. Finally the last step in ABA biosynthesis is catalyzed by aldehyde oxidases (AAO) localized in the cytosol. The transcripts of *ZEP*, *NCED3* and ABA 8′-hydroxylase enzymes are upregulated by ABA-mediated response [14]. Among the ABA catabolic pathways the C8′-methyl hydroxylation by cytochrome P450 monooxygenases (CYP707A) is considered as the major regulatory step. ABA level is thus the result of a balance between synthesis and degradation. External stresses like salt or drought as well as endogenous signals like hormones are known to regulate the transcription of the ABA biosynthetic or catabolic genes but little is known about the genes involved in the links between stress perception and modulation of ABA levels. Furthermore, stress-induced ABA accumulation regulates the global metabolic network in Arabidopsis [15,16].

It was previously shown that TOR inactivation results in starch accumulation or decrease in stress-related raffinose production [17–19]. Interestingly the levels of raffinose and certain amino acids increase in ABA treated Arabidopsis seedlings [20]. Furthermore, exogenous ABA treatment decreases starch accumulation [21].

In this article, we show that loss-of-function mutations in *Lst8-1* and *Raptor3g* genes or inhibition of the TOR complex activity cause a significant decrease in ABA level as well as in expression of the *ZEP*, *NCED3* and *AAO3* genes involved in ABA biosynthesis, in contrast to the ABA catabolic *CYP707A2* and *CYP707A3* genes which were induced. Our results provide one step forward in understanding the TOR and ABA signaling networks collaboration in plant growth and metabolism.

2. Materials and methods

2.1. Plant material

Seeds of the *lst8-1* (called thereafter *lst8*, SALK_02459) homozygous T-DNA mutant line (Supplementary Fig. 1A) were obtained from the Nottingham Arabidopsis Stock Centre (NASC) [7]. Two homozygous mutant lines *rap22* (SALK_022096) and *rap78* (SALK_78159) with T-DNA insertions in the *AtRaptor3g* (At3g08850) gene (Supplementary Fig. 1B), were obtained from NASC or the Arabidopsis Biological Resource Center (ABRC) [22].

2.2. Plant growth conditions

Seeds of mutant lines *lst8*, *rap22*, *rap78* and of wild type (Col-8) were surface sterilized by treatment with 1/10 (v/v) Bayrochlore/96% ethanol for 5 min and washed in 96% ethanol. Seeds were sown on Petri dishes containing 0.4 × MS medium with 1% sucrose, 0.8% agar, pH 5.8. After two days of stratification at 4 °C, plates were transferred in a controlled growth chamber with 16 h light (80–90 μE/m^2/s)/8 h night photoperiod, 18 °C. Then, five-day old Arabidopsis seedlings were transferred to vertical plates on the same medium and grown for 8 days.

2.3. AZD-8055 and ABA treatments

Five-day-old Arabidopsis seedlings grown as described above were transferred to vertical plates containing 0.4 × MS solid medium supplemented with 1% sucrose and either 1 μM and 2 μM of AZD-8055 or 10 μM and 25 μM of exogenous ABA or with the same amount of DMSO or 50%-ethanol respectively as control and grown for 8 days.

2.4. Root growth measurements

After transfer of five-day-old seedlings to vertical plates, the primary root growth was measured every two days. The vertical plates were scanned and the primary root length was estimated with the Image J software.

2.5. Germination test

Seeds of different genotypes were sown on 0.4 × MS solid medium with different concentration of ABA or 50%-ethanol as control. After 2 days of stratification at 4 °C, the plates were transferred to the same growth chambers as above. The number of germinated seeds (defined by a visible radicle protrusion) was scored at the indicated times.

2.6. Hormone measurements

For each sample, 100 mg of fresh powder were extracted with 0.8 mL of acetone/water/acetic acid (80/19/1 v/v/v). Abscisic acid, salicylic acid, jasmonic acid, and indole-3-acetic acid stable labelled isotopes used as internal standards were prepared as described in Ref. [23]. Two ng of each standard was added to the sample. The extract was vigorously shaken for 1min, sonicated for 1 min at 25 Hz, shaken for 10 min at 4 °C in a Thermomixer (Eppendorf), and then centrifuged (8000 g, 4 °C, 10 min). The pellets were re-extracted twice with 0.4 mL of the same extraction solution, then vigorously shaken (1 min) and sonicated (1 min; 25 Hz). After the centrifugations, the three supernatants (Final Volume 1.6 mL) were pooled and dried.

Each dry extract was dissolved in 100 μL of acetonitrile/water (50/50 v/v), filtered, and analyzed using a Waters Acquity ultra performance liquid chromatograph coupled to a Waters Xevo Triple quadrupole mass spectrometer TQS (UPLC-ESI-MS/MS). The compounds were separated on a reverse-phase column (Uptisphere C18 UP3HDO, 100*2.1 mm*3 μm particle size; Interchim, France) using a flow rate of 0.4 mL min^{-1} and a binary gradient: (A) acetic acid 0.1% in water (v/v) and (B) acetonitrile with 0.1% acetic acid. Mass spectrometry was conducted in electrospray and Multiple Reaction Monitoring scanning mode (MRM mode), in positive ion mode for the indole-3-acetic acid and in negative ion mode for the other hormones. Relevant instrumental parameters were set as follows: capillary 1.5 kV (negative mode), source block and desolvation gas temperatures 130 °C and 500 °C, respectively.

2.7. RNA extraction and quantitative real-time -PCR (RT-qPCR)

Total RNA was extracted from powder of each sample using TRIzol® Reagent (Ambion). Total RNA (1 μg) was used as a template to perform RT reactions using Moloney murine leukaemia virus reverse transcriptase (Thermo Scientific) and oligo(dT)15 primers (Thermo Scientific). Gene expression analysis were determined by RT-qPCR using 5 μl of a 1/10 dilution of first-strand cDNA with Takyon™ Rox SYBR® blue dTTP (Eurogentec) in a total volume of 15 μl Realplex MasterCycler Eppendorf®. All the experiments were performed following the manufacturers' instructions. Transcript levels were determined using specific primers (Supplemental Table 1). The relative expression of each gene was normalized to the level of a synthetic reference gene (SRG) using EF1α and APT1 as reference genes (%SRG = $\sqrt{2^{(CtEF1-Ctgoi)} \times 2^{(CtAPT1-Ctgoi)}}$) [24].