Short Communication

Construction of a rapamycin-susceptible strain of the unicellular red alga
* Cyanidioschyzon merolae * for analysis of the target of rapamycin (TOR) function

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Sousuke Imamura,1,2,* Keiko Taki,1,2 and Kan Tanaka1,2,*

1 Laboratory for Chemistry and Life Science, Institute of Innovative Research, Tokyo Institute of Technology, Yokohama, Japan
2 Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Saitama, Japan

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Abbreviations: 5-FOA, 5-fluoroorotic acid; DMSO, dimethyl sulfoxide, kbp, kilobase pair; kDa, kilodalton; PEG, polyethylene glycol

The target of rapamycin (TOR) is a serine/threonine protein kinase that plays a central role in the regulation of cell growth and metabolism (Laplante and Sabatini, 2012). This protein, which is structurally and functionally conserved among eukaryotes (Virgilio and Loewith, 2006; Wullschleger et al., 2006), is found in two functionally distinct multi-protein complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2). TORC1 regulates cell growth and metabolism in response to nutrient and energy requirements (Virgilio and Loewith, 2006; Wullschleger et al., 2006) and its functions are specifically inhibited by rapamycin (Heitman et al., 1991). TORC2, involved in the regulation of the cytoskeleton structure and spatial features of cell growth, is not inhibited by rapamycin (Virgilio and Loewith, 2006; Wullschleger et al., 2006).

Although rapamycin is a powerful tool to reveal the functions of TOR in cell growth and metabolism in eukaryotes, most land plants and algae do not show clear phenotypes against the rapamycin treatment (Crespo et al., 2005; Imamura et al., 2013; Sormani et al., 2007; Xiong and Sheen, 2012; Xiong et al., 2016) which obscures the functions of TOR in these plant lineages. To overcome this problem, we previously constructed a rapamycin-susceptible *Cyanidioschyzon merolae* F12 strain by expressing the *Saccharomyces cerevisiae* FKBP12 protein in cells of this alga (Imamura et al., 2013). To obtain the F12 strain, a plasmid harboring the *S. cerevisiae* FKB12 gene for constitutive expression in *C. merolae* was transformed into strain M4, a uracil-auxotrophic mutant (Minoda et al., 2004). Transformants were then selected using the UMP synthase gene as a marker for uracil prototrophy (Imamura et al., 2013). Using the *C. merolae* F12 strain, we recently revealed that TOR plays a central role in triacylglycerol accumulation in microalgae (Imamura et al., 2015).

Because of its simple cell architecture and genome, low gene redundancy, and capacity for gene-knockout by homologous recombination, *C. merolae* is considered to be a good model photosynthesizing eukaryote for understanding various fundamental molecular mechanisms (Imamura et al., 2009; Kuroiwa, 1998; Matsuzaki et al., 2004). Although these features also make *C. merolae* suitable for the analysis of TOR functions using the F12 strain, no selection marker is available in F12 enabling its use as a host. Consequently, the establishment of a novel *C. merolae* strain having both rapamycin-sensitive and uracil-auxotrophic phenotypes is required to further investigate TOR functions using a molecular genetic approach.

In a recent study, we demonstrated that the T1 strain, in which the *URA5.3* gene is completely deleted, is a 5-fluoroorotic acid (5-FOA)-resistant, backgroundless host for transformation experiments (Taki et al., 2015). In the study reported here, we constructed a plasmid pKTL1, serving as a genomic knock-in cassette for insertion into the *URA5.3* gene (Fig. 1a), and obtained a novel rapamycin-susceptible *C. merolae* strain in which the *URA5.3* gene is replaced by a DNA fragment for constitutive expression of *S. cerevisiae* FKB12 (hereafter referred to as the ScFKBP fragment) (Imamura et al., 2013). We then successfully used the isolated strain as a host strain.

*Corresponding authors: Sousuke Imamura and Kan Tanaka, Laboratory for Chemistry and Life Science, Institute of Innovative Research, Tokyo Institute of Technology, Yokohama, Japan; Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Saitama, Japan.
E-mail: simamura@res.titech.ac.jp kntanaka@res.titech.ac.jp

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to analyze TOR kinase activity in vivo.

The ScFKBP fragment was generated by PCR with primers pKTL1_ScFKBP_F1 (5′-AGATCTCATATGGATCTATCGCGGTGTTGAGAG-3′) and pKTL1_ScFKBP_R1 (5′-GCCCATTTAAGGTGCGTTCGATTGC-3′), with pKF-ScFKBP12 used as a DNA template (Imamura et al., 2013). All PCR amplifications in this study were carried out with KOD-Plus-Neo DNA polymerase (Toyobo). The PCR-amplified fragment was cloned into EcoRV/Hpa1-digested pKTL1 (Fig. 1a) using InFusion cloning (Clontech) to create pKTL1-ScFKBP12. pKTL1 was constructed in four steps as follows. First, to construct the plasmid pGL3_linker_plus, linker DNA was generated by annealing two synthetic oligonucleotides, Luclinker (Kpn)-Hd (5′-GACTAGTGCGCGCTCGCAGAGATCTCATATGgatatcT-3′; lowercase letters indicate EcoRV recognition sequences) and Luclinker(Hd)-(Kpn) (5′-AGCTATGatatcCATATGAGATCTCTGCA-GGGCGGCGCAGATGCTG-3′; lowercase letters indicate EcoRV recognition sequences); this linker DNA was inserted into the KpnI and HindIII site in plg3-Basic (Promega) to give the plasmid pGL3_linker_plus harboring an EcoRV site in the integrated synthetic fragment. Second, to obtain two fragments upstream (–2,761 to –586, +1 as the initiation codon) or downstream (1,393 to 3,394) of the URA5.3 locus, PCR was carried out using C. merolae genomic DNA as a template and the following primers: URA_-2761F (5′-GCCCACCAGCGGCTACG-3′) and URA_-586R (5′-TAAATATAATTTACAAAAATCATCCAG-3′) for the upstream fragment, and HpaI URA_1393F (5′-aactTTCCATATGAGCGAAGCGAAGCG-3′; lowercase letters indicate bases added to retain the Hpa1 site) and URA_3394R_Stul (5′-CCCGAggCCTGGGTTGAGGCGC-3′; lowercase letters indicate bases altered to insert the Stul site) for the downstream fragment. Third, to construct the plasmid pGL3_linker_ura-up, the URA5.3 upstream fragment was inserted into the blunted NcoI site of the plasmid pGL3_linker_plus. Finally, to construct the plasmid pKTL1, the URA5.3 downstream fragment was inserted into the Hpa1 site of the plasmid pGL3_linker_ura-up. Mighty Mix (Takara) was used in the each ligation step according to the manufacturer’s instructions. The ScFKBP fragment respectively harboring URA5.3 upstream and downstream regions at 5′ and 3′ ends was amplified by PCR with primers URA5′_F1 (5′-GCCCAACCAGCGGCTACGCTG-3′) and URA3′_R1 (5′-CCCGAggCCTGGGTTGAGGCGC-3′), with pKTL1-ScFKBP12 used as a DNA template. The resulting 7.1-kbp fragment was used in polyethylene glycol (PEG)-mediated transformation of wild-type cells. Transformants were then selected on modified Allen’s 2 (MA2) plates (Imamura et al., 2010) containing 5-FOA and uracil following the same methods used for isolation of the T1 strain (Taki et al., 2015).

After an approximately 2-month incubation period for selection, isolates were screened by PCR using each genomic DNA as a template and two primers: F1 (5′-GGAGGGCACCCTACGCTGAAAG-3′) and R1 (5′-GCCCATTAGGAGTGCGTTCGATTGC-3′), with pKTL1-ScFKBP12 used as a DNA template. The PCR products were resolved by 1.0% agarose gel electrophoresis. Positions based on a molecular size marker are indicated in kilobase pairs on the left.

Fig. 1. Construction of the Saccharomyces cerevisiae FKBP12 expression strain.

(a) Schematic representation of pKTL1. “URA5.3 up” and “URA5.3 down” respectively denote regions upstream and downstream of URA5.3. Luc indicates the coding region of the luciferase gene in pGL3-Basic. The position of primers URS5′_F1 and URS5′_R1, used for PCR amplification of the DNA fragment for the transformation experiment, are indicated by arrowheads. EcoRV and Hpa1 are unique restriction sites recognized by each restriction enzyme. Each two NotI and Stul recognition sequences is located at the 5′-end of URA5.3 up and the 3′-end of URA5.3 down. (b) Schematics for the URA5.3 locus in wild-type (upper) and SF12 (lower) strains. TAPCC, TAPCC, and FLAG denote the APCC terminator region, APCC, and the FLAG-tag, respectively. Arrows indicate URA5.3 and S. cerevisiae FKBP12 open reading frames. The positions of primers used for the PCR analysis shown in c–f are indicated by arrowheads. F1 and R2 anneal outside of the integration region. (c–f) Confirmation of the S. cerevisiae FKBP12 expression strain SF12. Genomic DNAs were used as templates for PCR analyses with primer sets F1/R1 (c), F1/R2 (d), F2/R3 (e), or F1/R4 (f). The PCR products were resolved by 1% agarose gel electrophoresis. Positions based on a molecular size marker are indicated in kilobase pairs on the left.
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to yield a 7.3-kbp fragment from the positive strain and a 6.3-kbp fragment from the parental wild-type strain (Fig. 1d);

2) F2 (5′-CAGTTT CATGCTTTTGGAGATTG-3′) and R3 (5′-ACCCTT GCCTTACAGCAGATA-3′), with no amplification expected from the positive strain and a 0.4-kbp fragment expected from the wild-type strain (Fig. 1e); and

3) F1 and R4 (5′-GTACGGCGTTCGAAAAATAGCG-3′), with 0.4-kbp fragments expected from both positive and wild-type strains (Fig. 1f).

As shown in Figs. 1c–f, the ScFKBP fragment was integrated as expected into the URA5.3 locus in the positive strain, the latter referred to as strain SF12.

To further confirm the replacement of URA5.3 by the ScFKBP fragment, we examined S. cerevisiae FKBP12 expression, uracil auxotrophy, 5-FOA resistance, and rapamycin-susceptible phenotypes of strain SF12. Immunoblot analysis with monoclonal anti-FLAG antibody (F3165; Sigma) was performed as described previously (Imamura et al., 2013, 2015), except that the lysis buffer for protein extraction contained phosphatase inhibitor cocktail (Nacalai Tesque, 100-fold dilution). This analysis confirmed the expression of the FLAG-tagged S. cerevisiae FKBP12 in the SF12 strain (Fig. 2a). As shown in Fig. 2b, SF12 exhibited uracil auxotrophy; in addition, it displayed 5-FOA resistance in a manner similar to the URA5.3 deletion mutant T1 (Taki et al., 2015). In agreement with our previous observation of strain F12 (Imamura et al., 2013, 2015), SF12 strain also exhibited rapamycin-susceptible phenotypes, such as growth inhibition (Fig. 2c) and lipid droplet accumulation (Fig. 2d) in the presence of rapamycin. Lipid droplets were detected with BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) as described previously (Imamura et al., 2015). Rapamycin-induced lipid droplet accumulation was not observed in strain T1 (data not shown). These results further support the conclusion that the URA5.3 gene was correctly replaced by the ScFKBP fragment.

Next, to evaluate whether the SF12 strain can be used as a host strain for transformation, we cloned human eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) into pSUGA (Fujii et al., 2013) to create pSUGA-4EBP1, which was then introduced into SF12 cells to generate a 4EBP1-expressing strain. We constructed this strain because our previous in vitro experiment demonstrated that C. merolae TOR can phosphorylate human 4EBP1 (Imamura et al., 2013). It should be noted that no 4EBP1 homolog is present in C. merolae based on the whole genome sequence information (Matsuzaki et al., 2004; http://merolae.biol.s.u-
The human 4EBP1 gene was amplified with primers 4EBP1_pSUGA_F1 (5’-CGTTCGTTGACCCCATGTCGGGGGCAGCAGC-3’) and 4EBP1_pSUGA_R1 (5’-GTCGACTCTAGACCCCTAATTGCTCATCTCAACTG-3’), with human 4EBP1 cDNA (HG10022-M; Sino Biological) used as a template. The amplified gene was then cloned into SmaI-digested pSUGA using InFusion cloning. Transformants were selected by uracil autotrophy because pSUGA harbored the UMP synthase gene (Fujii et al., 2013) as a selection marker. As a control experiment, pSUGA was also introduced into SF12 cells. Transformation with pSUGA-4EBP1 resulted in the growth of numerous colonies. Colony PCR analysis using 4EBP1_pSUGA_F1 and R1 confirmed the presence of the 4EBP1 gene in 12 independent strains (data not shown). We chose one of these strains, SF12-4EBP1, as a representative for further examination. We also isolated transformants using pSUGA and the selected one, SF12-pSUGA, for use as a control strain. To check for 4EBP1 expression in the transformant, we conducted an immunoblot analysis using anti-4EBP1 antibody (#9452; Cell Signaling Technology). As shown in Fig. 3a, two major bands of the predicted molecular size (about 18 kDa) were detected in the SF12-4EBP1 strain but not in SF12-pSUGA.

It has recently been indicated that several kinases phosphorylate 4EBP1 dependent on or independent of mammalian TOR (mTOR) (Qin et al., 2016). The phosphorylation of 4EBP1 by mTOR are well understood, and has been reported to occur in HEK 293 cells in the following order: phosphorylation of Thr 37/Thr 46 (numbered according to human 4E-BP1), followed by Thr 70 and finally Ser 65, with these phosphorylations dependent on mTOR activity (Gingras et al., 2001). This finding raises the possibility that the TOR-dependent phosphorylation status of 4EBP1 was responsible for the different band migration patterns observed above. Thus, we next subjected SF12-4EBP1 strain to 6 h of rapamycin or DMSO treatment followed by detection with anti-4EBP1 antibody and antibodies that specifically recognize the following phosphorylated amino acids of 4EBP1: phospho Thr 37 and/or Thr 46 (Thr 37/Thr 46) (#9459; Cell Signaling Technology) or phospho Ser 65 (#9451; Cell Signaling Technology). As shown in Fig. 3b, the upper band disappeared under TOR-inactivation or calf intestinal alkaline phosphatase (CIAP) treatment condition when anti-4EBP1 antibody was used. Furthermore, the effect of CIAP treatment followed by detection with anti-4EBP1 antibody and antibodies that specifically recognize the following phosphorylated amino acids of 4EBP1: phospho Thr 37 and/or Thr 46 (Thr 37/Thr 46) (#9459; Cell Signaling Technology) or phospho Ser 65 (#9451; Cell Signaling Technology). As shown in Fig. 3b, the upper band disappeared under TOR-inactivation or calf intestinal alkaline phosphatase (CIAP) treatment condition when anti-4EBP1 antibody was used. Furthermore, the effect of CIAP treatment followed by detection with anti-4EBP1 antibody and antibodies that specifically recognize the following phosphorylated amino acids of 4EBP1: phospho Thr 37 and/or Thr 46 (Thr 37/Thr 46) (#9459; Cell Signaling Technology) or phospho Ser 65 (#9451; Cell Signaling Technology). As shown in Fig. 3c, the band patterns were similar when anti-4EBP1 antibody was used (Fig. 3b). These results indicated that anti-phospho Thr 37/Thr 46 antibody recognizes 4EBP1 protein, but did not specifically recognize
phospho Thr 37 and/or Thr 46 in \textit{C. merolae} cells. In contrast, only the upper band was detected when anti-phospho Ser 65 antibody was used, and this band disappeared when cells were treated with rapamycin (Fig. 3d). The detected band with anti-phospho Ser 65 antibody was not visible in the total protein treated with CIAP, but the effect was blocked by the addition of phosphatase inhibitor cocktail.

These results indicate that TOR activity can be monitored in \textit{C. merolae} SF12-4EBP1 cells by observing the band migration pattern or Ser 65 phosphorylation status.

In conclusion, we have successfully constructed the rapamycin-susceptible \textit{C. merolae} strain SF12, in which the ScFKBP fragment has been replaced by the \textit{URA5.3} gene. Furthermore, we have demonstrated that SF12 can be used as a host strain for transformation experiments applicable to various research topics, such as the analysis of TOR-signaling regulators. As an example, SF12 was successfully applied for the construction of human 4EBP1-expressing strain SF12-4EBP1, in which TOR kinase activity can be monitored in vivo. Changes in TOR activity under various environmental and physiological conditions using SF12-4EBP1 would be an interesting future study focus, with the resulting data providing valuable insights into TOR functions in microalgae. Finally, the method used in this study—integration of a target gene into the \textit{URA5.3} locus—represents a novel strategy for the molecular genetic analysis of \textit{C. merolae}, as functional relationships between two different genes can be analyzed in vivo.

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References


