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添付資料：研究報告書

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1．　助成金額：600,000 円

2．　研究テーマ
分裂酵母における Ran　GTPase　新規変異体の単離と解析

3．　成果の概要
分裂酵母の温度感受性変異株（KP3272）を用い、関連遺伝子（抑圧遺伝子群）の分離と機能解析を行っている。現在まで、①*spi1*$^{+}$、*pim1*$^{+}$と SPAC6G9.14（*puf1*$^{+}$）三つの 相補遺伝子を取得した；　②*spi1*$^{+}$と *pim1*$^{+}$が KP3272 の温度感受性を 36 度まで、*puf1*$^{+}$が KP3272 の温度感受性を 34 度まで相補することを確認した；③KP3272 の表現型は *spi1-25* の表現型と違って、*pim1-46* の表現型と一致である。

4．　研究業績

① 学会における発表　　(無)・有（学会名・演題）

② 発表した論文　　(無)・有（雑誌名・題名）

— 日中医学協会助成事業 —

分裂酵母における Ran GTPase 新規変異体の単離と解析

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<Abstract>

Spi1p of *Schizosaccharomyces pombe* is a structural homolog of the mammalian GTPase Ran, and *spi1*$^+$ is an essential gene. In a screen designed to identify fission yeast genes required for living in high temperature(36℃), we identified a strain that carries a point mutation in the SpRan GTPase. Ran is an abundant Ras-like small GTPase, which is mainly localized in the nucleus[1], like other GTPases, switches between a GTP-bound and a GDP-bound form. The Ran family of GTPases, have been implicated in variety of nuclear processes including cell cycle progression, nucleocytoplasmic transport of protein and RNA, initiation of DNA replication, RNA metabolism, nucleolar and chromatin structure, and transcriptional regulation[2,3,4]. Ran and its structural and functional homologs interact with proteins that modulate its nucleotide-bound state: Guanine nucleotide exchange factors (GEFs) that stimulate the exchange of GDP for GTP and GTPase-activating proteins (GAPs) that catalyze the intrinsic GTPase activity, hydrolyzing the bound GTP to GDP. In fission yeast, the functioning of the spi1p GTPase system require a precise balance between the GDP- and GTP-bound forms of Ran GTPase spi1p, that the nucleotide-bound state of the GTPase correlates with its intracellular localization. Pim1p has been found in *S. pombe* the spi1p GEF and rna1p, has been shown to have GAP activity. Here, we describe the isolation and characterization of *spi1-93*, a mutant allele of the *spi1*$^+$ gene. The Ran guanine nucleotide GEF Pim1 mutant (*pim1-46*)[5] cells and *spi1-93* cells exhibited similar phenotypes. Overexpression of wild-type *spi1*$^+$ and *pim1*$^+$ suppressed the ts phenotype of *spi1-93* and *pim1-46* mutant cells and overexpression of SPAC6G9.14(*puf1*$^+$), pumilio-family RNA binding protein coding gene, only partially suppressed the TS phenotype of these two mutant cells. We also found another *spi1* mutant cells(*spi1-25*)[6] with different mutation site in *spi1*$^+$ gene showed different phenotypes with *spi1-93* and *pim1-46*, and the obtained two multicopy suppressor genes *pim1*$^+$ and *puf1*$^+$ didn' t suppress TBZ sensitivity of *spi1-25*. Further studies are needed to reveal the mechanism underlying the regulation of suppressants and more multicopy suppressor genes are needed to be identified in future.

<Key words>

Ran GTPase, *Schizosaccharomyces pombe*, GEF, GAP, temperature sensitive (TS)

<Introduction>

Spi1p of Schizosaccharomyces pombe is a structural homolog of the mammalian GTPase Ran, and *spi1*$^+$ is an essential gene. Ran is an abundant Ras-like small GTPase, which is mainly

localized in the nucleus, like other GTPases, switches between a GTP-bound and a GDP-bound form. The distribution between the GTP- and GDP-bound forms of the protein is regulated by evolutionarily conserved gene products, Rna1p and Pim1p, functioning as GTPase-activating protein (GAP) and guanine nucleotide exchange factor(GEF), respectively. Cells are sensitive to the balance between the two forms (spi1p-GDP/spi1p-GTP) of the GTPase. The Ran family of GTPases, have been implicated in variety of nuclear processes including cell cycle progression, nucleocytoplasmic transport of protein and RNA, initiation of DNA replication, RNA metabolism, nucleolar and chromatin structure, and transcriptional regulation. However, the primary role(s) of this system has yet to be determined, and many other questions relating to the functioning and regulation of the Ran system remain unanswered. Here, we describe the isolation of temperature mutant *spi1-93*, an allele of the *spi1*+ gene encoding a homolog of Ran GTPase and in addition, we also provided the results that the function of Spi1 was regulated by suppressants Guanine nucleotide exchange factors(GEF) Pim1 and pumilio-family RNA binding protein Puf1.

<Materials and methods>

Strains, media, and genetic and molecular biology methods— *Schizosaccharomyces pombe* strains used in this study are listed in Table 1. The complete medium YPD and the minimal medium EMM have been described previously[7]. Standard genetic and recombinant-DNA methods[8] were used except where noted.

Isolation of the spi1-93 mutant— The *spi1-93* mutant (KP3272) was isolated in a screen of cells that had been mutagenized with nitrosoguanidine as described previously[9]. To clone the mutated gene, the *spi1-93* mutant was grown at 27° C and transformed with a fission yeast genomic DNA library as described previously[9]. The Leu+ transformants were replica-plated onto YPD plates at 36° C, and the plasmid DNA was recovered from transformants that showed plasmid-dependent rescue.

These plasmids complemented the temperature sensitivity of *spi1-93* mutant. By DNA sequencing, the suppressing plasmids were identified to contain the *spi1*+ gene (SPBC1289.03c). The fragment of *spi1*+ gene from genome of KP3272 was amplified by PCR and was sequenced. We found the 69th glutamic acid (E) of Spi1p in KP3272 was mutated to lysine(K).

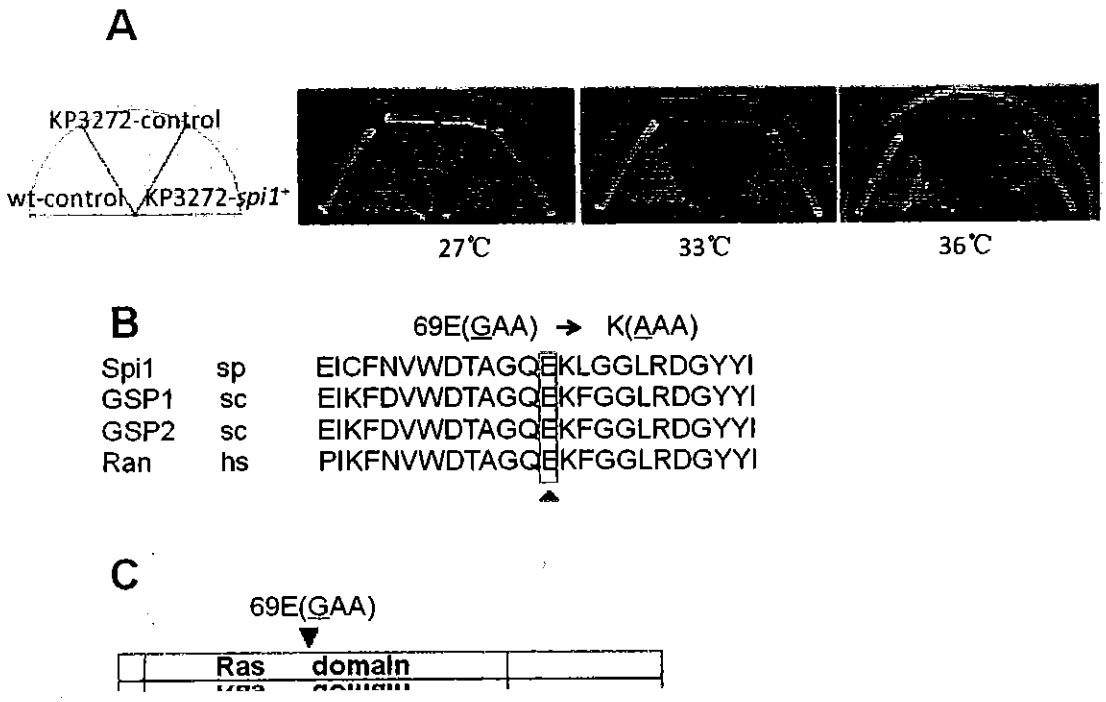
Isolation of the multicopy suppressor genes— To screen for other suppressors of the *spi1-93* mutation, the mutant was transformed with a fission yeast genomic DNA library, and the Leu+ transformants were replica-plated onto both YPD plates at 34° C , 35° C and 36° C. The plasmids that complemented the ts phenotype were recovered from the cells and the nucleotide sequences of the regions flanking the inserts were determined. By Southern blot analysis, the suppressing plasmids fell into two classes, with one class containing the Guanine nucleotide exchange factors(GEF) Pim1 encoding gene *pim1*+ (SPBC557.03c), and the other class containing the pumilio-family RNA binding protein Puf1 encoding gene *puf1*+ (SPAC6G9.14).

<Results>

1. Isolation of new *spi1-93* mutant cells.

On YPD plate at 27° C, KP3272 grew equally well as compared with that of the wild-type cells (Fig. 1A); however, at 33° C and 36° C, the cells showed temperature sensitivity (TS) phenotype. The suppressing plasmids was obtained by complementation of the ts growth defect of KP3272, and nucleotide sequencing revealed the plasmids contained the *spi1*$^+$ gene (SPBC1289.03c). The fragment of *spi1*$^+$ gene from genome of KP3272 was amplified by PCR and was sequenced. We found the 69th glutamic acid (E) of Spi1p in KP3272 was mutated to lysine (Fig. 1B, C).

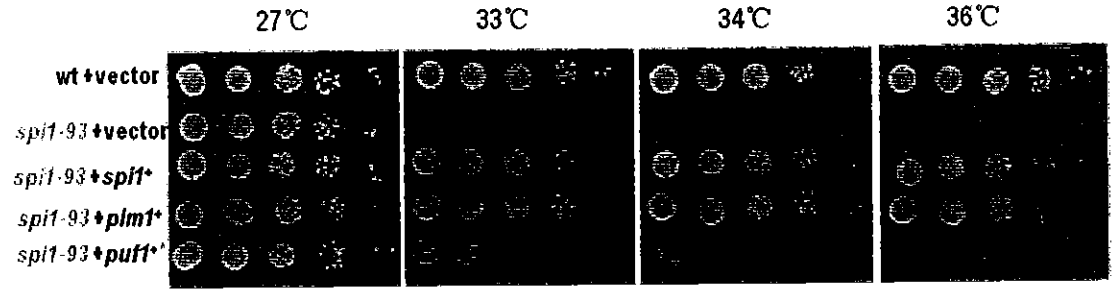
Fig. 1



2. *pim1*$^+$ (SPBC557.03c) and *puf1*$^+$ (SPAC6G9.14) are multicopy suppressor genes of *spi1-93*.

Overexpression of wild-type *spi1*$^+$ and Ran GEF encoding gene *pim1*$^+$ completely suppressed the ts phenotype of *spi1-93* mutant cells and overexpression of pumilio-family RNA binding protein coding gene, SPAC6G9.14 (*puf1*$^+$), only partially suppressed the ts phenotype of *spi1-93* mutant cells (Fig. 2).

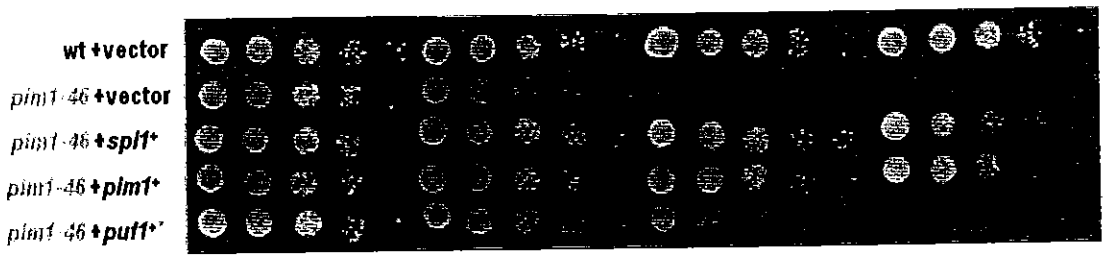
Fig. 2 *spi1-93*: *spi1*^{E69K}, the 69th glutamic acid (E) of Spi1p was mutated to lysine (K).



3. *pim1-46* exhibited similar phenotypes with *spi1-93*.

Overexpression of wild-type *spi1*$^+$ and *pim1*$^+$ also completely suppressed the ts phenotype of *spi1-93* mutant cells and overexpression of SPAC6G9.14(*puf1*$^+$) also partially suppressed the ts phenotype of *spi1-93* mutant cells(Fig. 3).

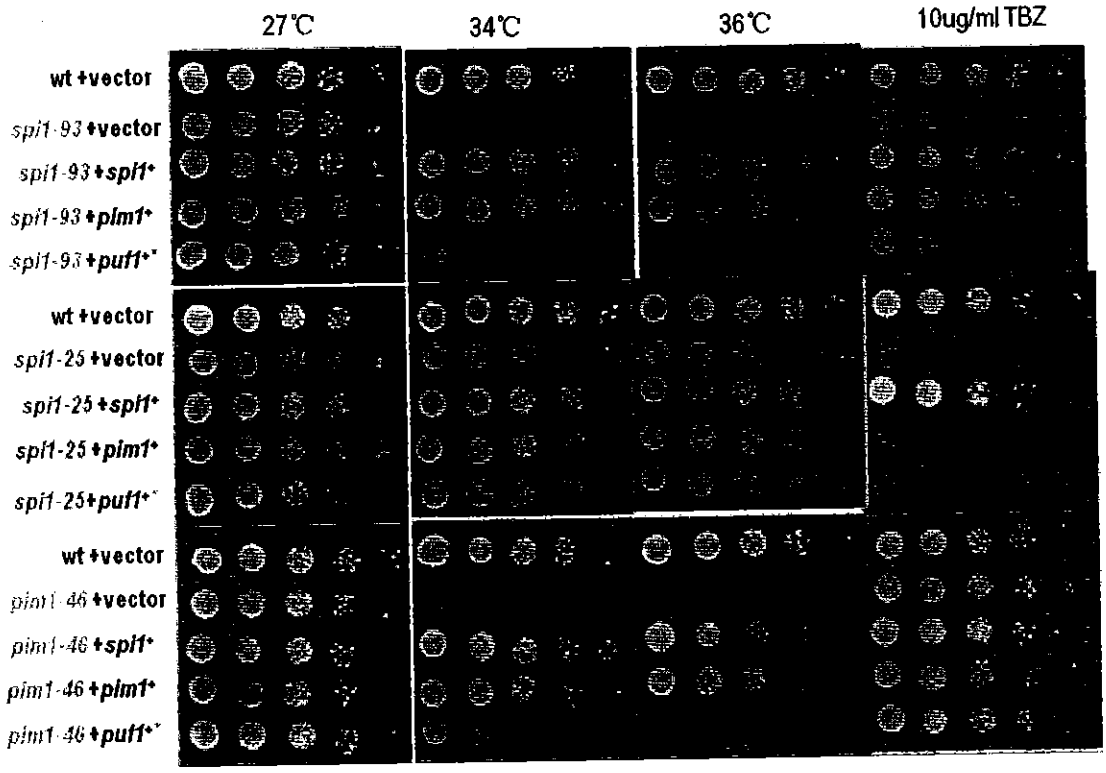
Fig. 3 *pim1-46*: *pim1*^{L113S}, the 113rd leucine (L) of Pim1p was mutated to serine(S).



4. *spi1-25* including different mutation site in *spi1*$^+$ gene exhibited different phenotypes with *spi1-93* and *pim1-46*.

spi1-25 exhibited thiabendazol(TBZ) sensitivity but not ts phonotype which is different with those of *spi1-93* and *pim1-46*. And we also found that overexpression of wild-type *pim1*$^+$ and SPAC6G9.14(*puf1*$^+$) didn't suppress the TBZ sensitivity of *spi1-25* mutant cells (Fig. 4).

Fig. 4 *spi1-25*: *spi1*^{V44I}, the 44th valine (V) of Spi1p was mutated to isoleucine (I).



<Discussion>

In our present study, we have identified a temperature-sensitive mutant *spi1-93* that is allelic to the *spi1*$^+$ gene encoding Ran GTPase, an abundant Ras-like small GTPase, which is mainly localized in the nucleus. We showed here that overexpression of wild-type *spi1*$^+$ and Ran GEF encoding gene *pim1*$^+$ completely suppressed the ts phenotype of *spi1-93* mutant cells and overexpression of pumilio-family RNA binding protein coding gene, SPAC6G9.14(*puf1*$^+$), only partially suppressed the ts phenotype of *spi1-93* mutant cells. And we also found that *spi1-93* exhibited similar phenotype with *pim1-46* but different with *spi1-25*. In future , we will continue to look for other multicopy suppressors of *spi1-93* mutant cells by screening to clearify the relationships between the identified multicopy suppressors and Ran GTPase Spi1. We will also investigate the interactions of Ran GTPase or mutated Ran GTPase with RanGEF or mutated RanGEF using the method of immunoblot.

Table1. Strains used in this study.

Strain	Genotype
KP3272	*h⁻ leu1-32 spi1-93 (spi1^{E69K})*
KP3842	*h⁻ leu1-32 pim1-46(pim1^{L113S})*
KP4113	*h⁻ leu1-32 spi1-25(spi1^{V44I})*

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