Original Article

Identification of *Schizosaccharomyces pombe ird* Mutants Resistant to Glucose Suppression and Oxidative Stress

(glucose suppression / oxidative stress / signalling / *Schizosaccharomyces pombe* / fission yeast)

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Abstract. Glucose is both the favourite carbon and energy source and acts as a hormone that plays a regulating role in many biological processes. Calorie restriction extends the lifespan in many organisms, including *Schizosaccharomyces pombe,* while uptake of high glucose leads to undesired results, such as diabetes and aging. In this study, sequence analysis of *Schizosaccharomyces pombe ird5* and *ird11* mutants was performed using next-generation sequencing techniques and a total of 20 different mutations were detected. *ird11* is resistant to oxidative stress without calorie restriction, whereas *ird5* displays an adaptive response against oxidative stress. We selected nine candidate mutations located in the non-coding (6) and coding (3) region among a total of 20 different mutations. The nine candidate mutations, which are thought to be responsible for *ird5* and *ird11* mutant phenotypes, were investigated via forward and backward mutations by using various cloning techniques. The results of this study provide report-like information that will contribute to understanding the relationship between glucose sensing/signalling and oxidative stress response components.

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Abbreviations: 2-DOG – 2-deoxyglucose, ARS – autonomously replicating sequence, EMS – ethyl methanesulphonate, PCR – polymerase chain reaction, PKA – protein kinase.

Introduction

Glucose plays an important role in the regulation of expression of genes involved in glucose sensing and signal transduction, transport, use of alternative carbon sources, spore formation, and stress response pathways (Carlson, 1999; Johnston, 1999; Rolland et al., 2001; Chen et al., 2003, 2008). In *Schizosaccharomyces pombe*, unlike *Saccharomyces cerevisiae*, the regulatory mechanisms in glucose sensing and signal transduction pathways are not fully understood, but intracellular signal transduction is sensed by Git3, which is a G protein-coupled receptor, via cyclic AMP (cAMP)-dependent protein kinase (PKA) (Welton and Hoffman, 2000; Hoffman, 2005). Two hexose transporters (Ght7 and Ght8-mitochondrial), which were identified by BLAST analysis, are also known, along with six different hexose transporter proteins (Ght1-Ght6) identified by Heiland et al. (2000).

In addition, it is known that high resistance to oxidative stress under glucose starvation conditions is provided by either activation of stress response genes due to carbon stress (Madrid et al., 2004), or a defence mechanism against the increased accumulation of reactive oxygen species (ROS) arising from aerobic respiration (Roux et al., 2009).

2-Deoxyglucose (2-DOG) is a synthetic glucose analogue that can be taken into cells and interfere with glycolysis and ATP production. Non-metabolized 2-DOG is taken up into the cell by the glucose transport system and is phosphorylated to 2-DOG-6-phosphate by yeast hexokinases without being metabolized (Novak et al., 1990). Studies with 2-DOG are not just about understanding the glucose metabolism. *S. cerevisiae* and *S. pombe* are Crabtree positive yeasts, and this effect is similarly seen in cancer cells. Studies on 2-DOG resistance in yeast models are important to understand the relationship between metabolism and cancer (Vishwanatha and D'Souza, 2017). Additionally, 2-DOG is also used as an anti-cancer agent (Laussel and Léon, 2020).

In our previous studies, *S. pombe* invertase repression defective (*ird*) mutants were clonally obtained by mutagenizing wild-type *S. pombe 972h*$^{-}$ with ethyl methanesulphonate (EMS) and selected on the basis of resistance to repression in the presence of 2-DOG. It was found that these mutants were two different complementation groups according to random spore analysis. As is known, the glucose consumption rate has an effect on glucose repression. Initial characterization of these mutants revealed that glucose repression of *ird5* mutant was higher than that of *ird11* mutant, although the glucose consumption rate of this mutant was lower than that of *ird11* mutant (Kig et al., 2005). The following studies revealed that *S. pombe ird11* mutant was always resistant to oxidative stress, irrespective of calorie restriction, while *S. pombe ird5* mutant exhibited an adaptive stress response due to the glucose deficiency (Palabiyik et al., 2012, 2013). In another study using *ird* mutants as models, the lifespan extension seemed to be related to glucose sensing/signalling rather than to the oxidative stress response and trehalose accumulation (Palabiyik and Ghods, 2015). These studies aiming to clarify the possible relationships between the glucose signalling, oxidative stress and lifespan in *S. pombe* using these mutants as a model system indicated the complexity of the control mechanisms responsible for glucose repression in *S. pombe.* Mutational analysis appears to be a good approach for dissecting the large numbers of regulatory factors that are involved in the different steps of the glucose sensing and signalling pathways in *S. pombe*.

The *S. pombe* genome can be easily altered by homologous recombination, and it can also undergo non-homologous recombination. Integration into an auxotrophic locus can be achieved with any plasmid that does not contain an autonomously replicating sequence (ARS) and contains the appropriate sequence. For example, a plasmid containing a related construct can be integrated into leu1-32 (Keeney and Boeke, 1994). Whole gene disruption or displacement (knock-out/knock-in) requires additional planning. High integration efficiency occurs using homologous sequences of more than 300 bp (Krawchuk and Wahls, 1999). Homology of less than 100 bp may also be used (Bähler et al., 1998), but the rate of non-homologous recombination increases compared to *S. cerevisiae*. The success of homologous integration depends on the specific gene and chromatin content. In addition, fragments or plasmids without replication origin may sometimes remain as unstable concatemers.

Chemical-induced mutagenesis contributes to the determination of the functions of genes and the effects of mutation by creating genomic variation. Induced mutations have been studied in rice and wheat (Henry et al., 2014). Blumenstiel et al. (2009) performed EMS-induced mutations in *Drosophila melanogaster* and examined the effects of these mutations on eggshell morphology by comparing the whole-genome sequence analysis of wild-type and mutant genomes. Genetic mapping of mutations in model systems was used to find genes that play a role in many basic biological processes, including human diseases (Leshchiner et al., 2012), and various studies aimed to determine the mutations that cause the disease by sequencing candidate genes (Evilä et al., 2016). Also, SNP variations were identified by next-generation sequencing in *C. elegans* (Doitsidou et al., 2010), mouse (Arnold et al., 2011) and *Arabidopsis thaliana* (Mokry et al., 2011). The next-generation sequencing technology was used in clinical and research applications of mutation analysis, and mutations in cancer-related genes were determined using this approach (Roychowdhury et al., 2011; Shao et al., 2016). Genomic variations were identified more easily with the next-generation sequencing technology (Salk et al., 2018).

In this study, we aimed to contribute to elucidation of the relationship between glucose sensing/signalling and oxidative stress response pathways. In this context, to determine the mutation leading to glucose repression and oxidative stress resistance phenotype in *S. pombe ird5* and *ird11* mutant strains, sequence analysis of mutants was performed using the next-generation sequencing technology (MiSeq System Illumina, Illumina Inc., San Diego, CA). According to the results, 20 different mutations in the *ird5* and *ird11* mutants were detected by comparing with wild-type reference genome *972h*$^{-}$. Then, nine candidate mutations in protein-coding (3) and non-coding regions (6), which are thought to be responsible for *ird5* and *ird11* mutant phenotypes, were analysed by forward and backward mutations using various cloning techniques.

Material and Methods

Strains and media

Bacterial and yeast strains used in this study are listed in Table 1. Standard *S. pombe* media YEA, EMM were used for culturing cells (Gutz et al., 1974, Moreno et al., 1991). YEA-DOG (0.5% yeast extract, 3% sucrose and 400 µg/ml 2-DOG) were used as selective medium for *S. pombe ird* mutants (Kig et al., 2005). The bacterial strain was grown on Luria-Bertani, LBA medium (Sambrook et al., 1989). Selection of bacterial transformants was done by using LBA supplemented with ampicillin (50 mg/l).

DNA isolation and whole-genome sequencing

S. pombe genomic DNA isolation was performed according to the method developed by Bähler et al. (1998). Selection of the mutant cells was carried out in media containing 2-DOG. After the cells were produced at 30 °C and 180 rpm overnight in 5 ml of YEL medium, they were harvested by centrifugation (1000 *g*).

The cells were suspended in 0.2 ml of lysis buffer (2% Triton X-100, 1% SDS glacial acetic acid, 100 mM NaCl, 10 mM Tris, 1 mM EDTA (pH 8.0)). Then, 0.2 ml of phenol:chloroform:isoamyl alcohol (25:24:1) and 0.3 g of acid-washed glass beads were added and homo-

Table 1. Bacteria and S. pombe strains and their genotypes used in the study

Strain	Vector	Genotype	Source
972	–	h^-	
ura4-D18	–	h^+/*ura4-D18*	
ird5	–	h^-/?	Kig et al., 2005
ird5u	–	h^-?/*ura4-D18*	This study
ird11	–	h^-/?	Kig et al., 2005
ird11u	–	h^-?/*ura4-D18*	This study
Mam301Δ	–	h^-/*M210 ura4-D18 leu1-32, SPCC4B3.03c:kanMX4*	Bioneer
DH5α	–	*dlacZ Δ M15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1)*	

genized by a dismembrator (Sartorius Mikro-Dismembrator S, Göttingen, Germany) for 4 min at 3000 rpm. The mixture was centrifuged, and the supernatant was removed and centrifuged again (16,000 *g*) by adding 1 ml of EtOH. The pellet was dissolved in 50 µl of water and stored at −20 °C. Genomic DNA concentration and purity were determined by nanodrop (ThermoScientific Nanodrop 2000, ThermoFisher Scientific, Vilnius, Lithuania).

Whole-genome sequencing of *S. pombe* strains was carried out in the "Illumina MiSeq System" (Illumina Inc.). To determine the differences between mutant strains and wild-type strains, whole-genome sequencing of each DNA sample was performed by the next-generation sequencing system and then, the mutations that were found in the genomes were identified.

In this study, an Illumina Nextera XT Library Preparation Kit was used to construct DNA libraries, and MiSeq System Illumina and a next-generation sequencing platform were used. During the library preparation process, the manufacturer's instructions were followed (Illumina Nextera XT Library Preparation Kit, Illumina Inc.). Agencourt AMPure XP (Beckman Coulter, Beverly, MA) was used during the product purification steps for library preparation. Sequencing was carried out with 2 × 150 bp readings.

The obtained data were aligned according to the reference genome, the *Schizosaccharomyces pombe* genome (GenBank ID: CU329670.1, CU329671.1 and CU329672.1) from NCBI (National Center for Biotechnology Information) was used as the reference genome during the alignment process (Wood et al., 2012). Alignments to three different chromosomes in the reference genome were performed using the BWA mem v.0.7.12 program (Li, 2013). After the alignment, data were filtered according to the quality control with program SAMTools v.1.2.0.

For analysis, adjustments of in/del regions with realignment, recalibration of scores of DNA sequence quality, optimization of parameters for the variations in the ordered sequences, and completion of the annotation process for the obtained variation list were performed. Then, variation lists were filtered according to the strand bias by taking the upper limit (20 %), and unreliable variations (< 80 %) were eliminated according to the detected variation percentage. The GATK v.3.3.0 program was used to determine the differences between the reference genome and samples. Subsequently, variant differences between mutant strains and the wild type were detected by the CLC Genomics Workbench program (CLCbio, Aarhus, Denmark).

Obtaining double mutants in S. pombe

To obtain the double mutants of *S. pombe* (*ird5h*$^-$ and *ird11h*$^-$) and *ura4h*$^+$, the cells of each strain were crossed in the SPA media and incubated for three days at 25 °C, and then ASCUS were separated by the de Fonbrune-type micromanipulator. For genotyping, they were plated onto selective media (MMA, MMA+uracil, MMA+uracil+2-DOG), then the mating type of double mutant cells was determined by crossing with the wild-type cells (Gutz et al., 1974).

Plasmids

In this study, pSGP572 (9.3 kb) allowing GFP to be fused to the C-terminus, commercially available from the National BioResource Project – Yeast (NBRP, Osaka City University, Japan) in Osaka, Japan was used as expression vector. pSGP572 includes a regulatable promoter of the *nmt1* gene, whose expression can be suppressed by thiamine, and the *ura4*+ gene for *S. pombe* and amp^R gene for *E. coli* as a marker gene (Maundrell, 1993). Also, a Thermo Scientific CloneJET PCR Cloning Kit (ThermoFisher) was used for cloning the selected coding and non-coding DNA fragment.

Cloning of targeted protein-coding and non-coding regions

We investigated whether the candidate mutations were responsible for the formation of *ird*5 and *ird11* phenotypes. For this purpose, we applied both forward and backward mutation strategies. The lithium acetate method of Gregan et al. (2006) was used for transformation of the prepared plasmids and cassettes into *S. pombe* cells.

To check all target genes by resulting sequence analysis, the polymerase chain reaction (PCR) was carried out with the Thermo Scientific Phusion High-Fidelity (ThermoFisher) DNA Polymerase enzyme by using *S. pombe 972h*$^-$ wild-type genome DNA as a template. Appropriate primers were designed by using the primer design tool "Integrated DNA Technologies" (Integrated DNA Technologies, Inc., Coralville, IA) for reconstruction of candidate DNA mutations responsible for the formation of *S. pombe ird5* and *ird11* phenotypes. The primers given in Table 2 were used for amplification of target genes or extragenic regions.

To construct pSGP572-Ssn6T, pSGP572-Ssn6D and pSGP572-Mam301T, oligonucleotide primers SSN6-TF/SSN6-TR, SSN6-DF/SSN6-DR, and MAM301-F/MAM301-R (Table 2) were used to amplify a 3309 bp fragment containing the complete *ssn6*$^+$ coding sequence, a 2274 bp fragment containing the lacking *ssn6Δ* (1-2274) coding sequence, and a 2040 bp fragment containing the complete *mam301*$^+$ coding sequence, respectively, from *S. pombe* genomic DNA. All three amplified fragments were digested with *Sal*I and *Bam*HI and ligated into the corresponding sites in pSGP572 (Pasion and Forsburg, 1999) to generate the recombinant plasmids (Fig. 1).

To perform backward mutations, while ird5u cells were transformed with pSGP572-Ssn6T and pSGP572-Ssn6D, ird11u cells were transformed with pSGP572-Mam301T, and transformants were selected on EMM plates containing adenine and leucine but lacking uracil. To test the growth following glucose repression, transformants were grown on EMM containing 2-DOG for 3 to 5 days at 30 °C. Also, pSGP572-Mam301T was transferred to the *S. pombe mam301Δ* strain.

To perform forward mutations, wild-type *S. pombe* (972h$^-$) cells were transformed with both pJET1.2 cloning vector that contains the mutant variants of *cip2*, *met7, rad25*, SPNCRNA.1063, *mam301, ssn6* and cassettes including mutant variants of SPBTRNAASN.01, SPAC1D4.11c and 1642507/8 Chr III.

Cloning of mutant fragments of target genes

SPAC19G12.16c (*cip2*-2644C>T), SPBC1709.17 (*met7*-2218C>T), SPAC17A2.13c (*rad25*-605C>T), SPNCRNA.1063-821G>A, SPCC4B3.03c (*mam301*-1286G>A), SPBC23E6.09 (*ssn6*-2272C>T), were PCR-amplified from strain *972h*$^-$ genomic DNA using oligonucleotides CIP2-F/CIP2-R, MET7-F/MET7-R, RAD25-F/RAD25-R, SPNCRNA.1063-F/SPNCRNA.

Table 2. Primer sequences used in the study and their melting temperatures (Tm °C)

	Forward primers		**Reverse primers**	**Tm (°C)**
SSN6-TF	TTTTGTCGACATGCCCCAATCACA AGTCGCT	SSN6-TR	AAGGATCCTTAAACTGACACGGTTT CTTT	50
SSN6-DF	TTTTGTCGACATGCCCCAATCACA AGTCGCT	SSN6-DR	AAGGATCCTTAACCGGTAGCAGAAG GAAG	54
SPBTRNAASN.01-F	ACTCGCATTTGCTGTCAATTT	SPBTRNAASN.01-R	TTGGTTATTGCTCACGGACTG TTAA	55
SPBTRNAASN.01-F1	–	SPBTRNAASN.01-R1	GCATTATAGGTCGGGTAGCATAG	51
CIP2-F	CCCAGCTTGTTCACGTTAGT	CIP2-R	GCCGGCAAATAGCCAATAGA	47
CIP2-F1	ATTATTTTTTTTCGCACTGTTTTTA GTGGATACTTAATGATTTTGTCTAA	CIP2-R1	TACAGCAGATACGAGATACGTTTAG ACAAAATCATTAAGTATCCACTAAAA	45
1642507/8 Chr III-F	AAATGCTAAAGGCCGCTAAAG	1642507/8 Chr III-R	RTAATCGCTTTTTTTTGATGTTTTT TGT	42
	–	1642507/8 Chr III-R1	AGTTGTTGATGCACCATTGAA	43
MET7-F	TGGCAAACCTATCAGTCAAGAG	MET7-R	ACCAATCATATCCGGCGTTAAT	46
MET7-F1	GCCAATGTATTTTCGATTCTTGAC	MET7-R1	AGTGTCAAGAATCGAAAATACAT	45
RAD25-F	CGATTGAACTGCCTTGATTGTC	RAD25-R	GCGAAGAAGCTGCATGATTAAA	49
RAD25-F1	TTCATCTCTCTTTTTTGTTTTTGC	RAD25-R1	AACAAAAAAGAGAGATGAAAGAA	49
SPAC1D4.11c-F	TGTAGGGTGCAACAGTAAAGAG	SPAC1D4.11c-R	GATGGTGATTGTTGGGTTGTTC	48
SPAC1D4.11c-F1	AATGGGGGGGGTAAAACAACAA AGCA	SPAC1D4.11c-R1	CCCCCCCCATTGGAATTACATTCTGT	48
SPNCRNA.1063-F	CCAAACAATCCCTATCCTCTTCT	SPNCRNA.1063-R	CCAGATTCCCGTACCTTGATATT	50
SPNCRNA.1063-F1	TTAAGGTTGTTTACTGAACTAT CCG	SPNCRNA.1063-R1	CAGTAAACAACCTTAATTGTGTCG	41
MAM301-F	ACGCGTCGACATGTCCCTATTGA GAATT	MAM301-R	CGGGATCCTTTCTTGCTTTTACCTTT	47
MAM301-F1	TACAAGAAAAGCATGCCGAGAG	MAM301-R1	TCTTGTAAATCCAAAACAGCTG	44
SSN6*-F	GCTGTGTATCGTGATGGTAGAA	SSN6*-R	GAGGAGACTTGGTAGACGAAATAG	47
SSN6*-F1	TACCGGTTAATCAGGCGTCGTG CAA	SSN6*-R1	CGCCTGATTAACCGGTAGCAGAA	50

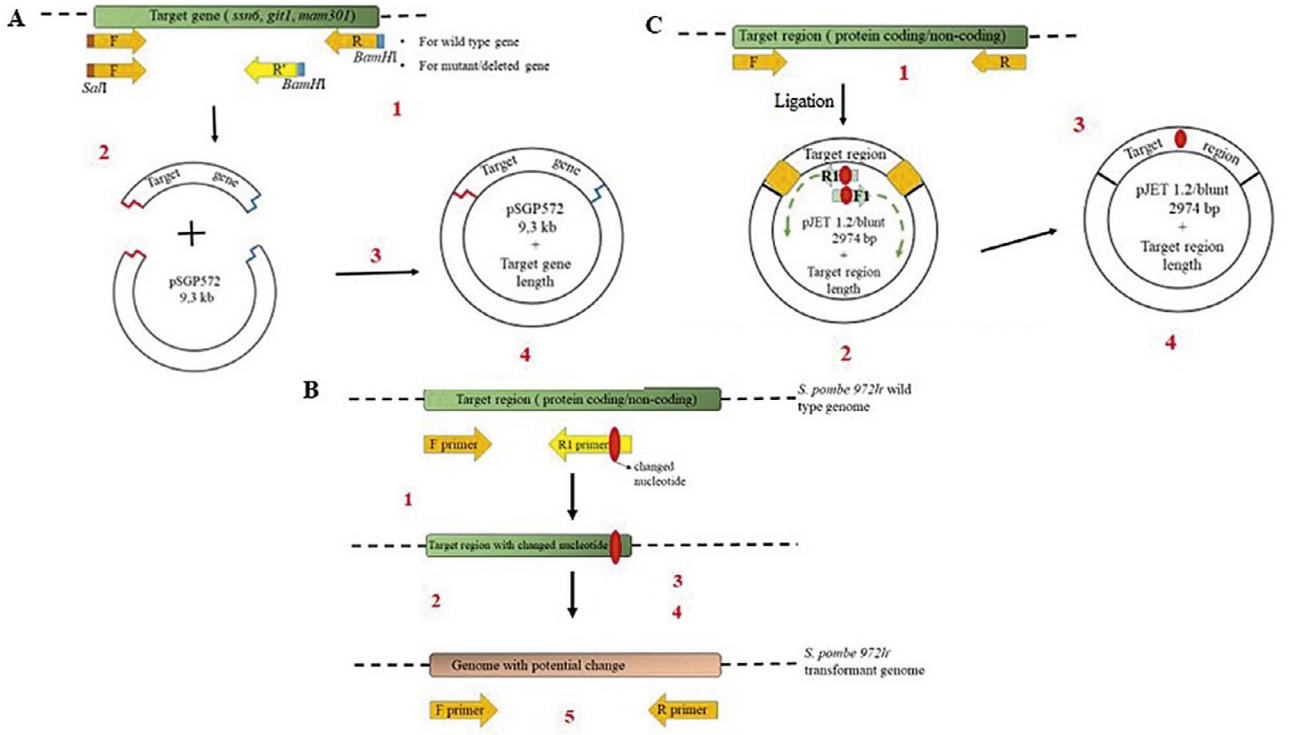

Fig. 1. Different cloning techniques used in this study. **A**. After amplification of the target gene, both the PCR product and the plasmid are cut by appropriate restriction endonucleases. Once the plasmid containing the target gene is formed by ligation, transformation is performed. (1: amplification of the target region by PCR; 2: cutting of PCR product and pSGP572 plasmid with *Sal*I and *Bam*HI restriction endonucleases; 3: ligation; 4: transformation of pSGP572 plasmid with the wild-type gene/mutant gene to *S. pombe* and selection) **B**. Using primers containing altered nucleotides, the target region is amplified by PCR and transformation into the cell is performed. (1: PCR; 2: transformation to wild-type *S. pombe 972h*$^-$; 3: selection of transformants; 4: genomic DNA isolation; 5: PCR for sequencing) **C**. The target region is amplified by PCR and, after cloning with the ThermoScientific pJET1.2 / blunt cloning kit, this plasmid is amplified with primers containing the altered nucleotides. After ligation, the plasmid containing the altered gene is transformed into the cell (1: amplification of the target region by PCR; 2: ligation of the target sequences into the pJET 1.2/blunt cloning vector; 3: production of a vector with a changed nucleotide by PCR using the pJET 1.2/blunt cloning vector including target sequences and primers R1 and F1; 4: transformation of pJET 1.2/blunt cloning vector with the changed nucleotide to wild-type *S. pombe* and selection).

1063-R, MAM301-F/MAM301-R, SSN6*-F/SSN6*-R, respectively. The PCR product was cloned into vector pJET1.2 (Thermo Scientific CloneJET PCR Cloning Kit, Vilnius, Lithuania) and used to transform *Escherichia coli* according to the manufacturer's instructions. Plasmids from *E. coli* transformants were purified. Plasmids containing the mutant alleles of target fragments were formed by amplifying with a second set of primers (CIP2-F1/CIP2-R1, MET7-F1/MET7-R1, RAD25-F1/RAD25-R1, SPNCRNA.1063-F1/SPNCRNA.1063-R1, MAM301-F1/MAM301-R1, SSN6*-F1/SSN6*-R1, respectively, Table 2) prepared for each gene. After these amplifying reactions, ligations of vectors were performed with T4 ligase (T4 DNA Ligase Invitrogen by ThermoFisher Scientific) (Fig. 1) and these pJET1.2 recombinants were transformed into *S. pombe 972h*$^-$ wild type. Transformants were selected on YEA and YEA with 2-DOG media.

Formation and transformation of plasmids and cassettes

Sequences of SPBTRNAASN.01, SPAC1D4.11c and sequences including the changes in nucleotide 1642507/8 on chromosome 3 were amplified by PCR and then these constructs were directly transformed to the *S. pombe 972h*$^-$ wild type in linear form (cassette structure) (Fig. 1). The constructs of SPBTRNAASN.01, SPAC1D4.11c and 1642507/8 Chr III include 335 nt, 869 nt and 220 nt homology with the wild-type genome, respectively. Transformants were selected on YEA and YEA with 2-DOG media.

Results and Discussion

Glucose sensing and signalling in *S. pombe* is regulated by the cAMP-dependent protein kinase A (PKA) pathway (Hoffman, 2005). However, the regulatory mech-

anism has not yet been fully elucidated. In this context, with the studies initiated in our research group, firstly mutants resistant to glucose suppression, termed *ird* mutants, were obtained by using EMS. In our further studies, it was found that *ird5* and *ird11* mutants were also resistant to oxidative stress and had extended lifespan (Kig et al., 2005; Palabiyik et al., 2012, 2013; Palabiyk and Ghods, 2015). Therefore, the *ird* mutants needed to be identified by whole-genome sequencing. As is known, whole-genome sequencing is a reliable method to identify mutations.

According to the next-generation sequencing platform, MiSeq System Illumina, 12.3 M reads and 6 Gb of data were obtained as 2 × 150 bp reads for three samples. The raw data generated from the reads were obtained in the FastQ format, and quality control of FastQ data was performed by the FastQC program. The number of readings and amount of data obtained for each sample are given in Table 3. In next-generation sequencing studies, the read depth is the number of high-quality reads of DNA fragments in the sequencing library of the samples before or after alignment to the reference. The read depth and the number of different DNA fragments sequenced are positively correlated. The minimum read depth required for sequencing varies depending on many biological factors (Sims et al., 2014).

Raw data were aligned to the three different chromosomes of the *S. pombe* genome (GenBank ID: CU329670.1, CU329671.1 and CU329672.1). The variants obtained by considering nucleotide and amino acid changes in the coding region, DNA localizations, types, changes, and read depths between mutant strains (*ird5, ird11*) and wild type (*927h⁻*) are given in Table 4 and Table 5.

After next-generation sequencing, a deletion and six single nucleotide variations on chromosome 1, an insertion and two single nucleotide variations on chromosome 2, and an insertion on chromosome 3 were detected in the *ird5* mutant (Table 4). A deletion and five single nucleotide variations on chromosome 1, an insertion and a single nucleotide variation on chromosome 2, and three single nucleotide variations on chromosome 3 were found in the *ird11* mutant (Table 5). Similar differences in *ird5* and *ird11* were detected at different alteration depths. These alterations may be due to the formation of the *ird* mutants.

We selected nine candidate DNA mutation regions in this study. The location and type of mutations in the targeted protein-coding and non-coding regions (SPAC 19G12.16c (*cip2*), SPNCRNA.1063, SPBC1709.17 (*met7*), SPBC23E6.09 (*ssn6),* 1642507/8 Chr III, SPAC 1D4.11c, SPAC17A2.13c (*rad25),* SPBTRNAASN.01, SPCC4B3.03c (*mam301)*) are given in Table 6.

Five of these selected regions are located in the regulatory, one of them in the intergenic, and three of them in the protein-coding region. Selected genes are potential candidate genes that are both human orthologues and may be related to regulation. It is thought that the altered phenotype in *ird* mutants may be associated with mutation(s) in the regulatory region(s). The reason why we have focused on the regulatory regions is that these *ird* mutants could not be identified with the complementation-based classical method in our previous studies. Considering that this may be caused by reasons such as the location of the mutation in the mutants (non-coding region, etc.) or other additional mutations being carried together, the non-coding regions from the candidate mutants determined in the sequence analysis were emphasized.

In previous studies, mutants resistant to glucose repression were selected by using 2-DOG in different organisms such as *S. cerevisiae* (Novak et al., 1990;

Table 3. Read counts, data and mean read depths of samples

Samples	Read Count (2 × 150)	Data	Mean Read Depth
972h⁻	3213644	1.649 GB	37
ird5	2835568	1.455 GB	31
ird11	5482099	2.813 GB	54

Table 4. Variants located in the S. pombe ird5 strain and not in the 927h⁻ wild type

Chromosome	Location	Type	Change	Read Depth	Coding Region Change	Amino Acid Change
CU329670	342539	SNV	C>T	20	–	–
CU329670	3528840	SNV	A>G	19	CAB11043.1:c.480A>G	–
CU329670	4413184	SNV	G>T	42	CAB66469.1:c.736C>A	–
CU329670	5070354	SNV	A>G	59	CAB60232.1:c.659A>G	CAB60232.1:p.Tyr220Cys
CU329670	5267920	SNV	G>A	33	–	–
CU329670	5327352	Deletion	G>–	55	CAB55174.1:c.487delC	CAB55174.1:p.Gln163fs
CU329670	5503081	SNV	G>A	23	CAB60016.1:c.507G>A	–
CU329671	1133028	SNV	C>T	37	CAA21256.1:c.504C>T	–
CU329671	1520665^1520666	Insertion	–>A	21	–	–
CU329671	3865776	SNV	C>T	62	CAA18877.1:c.2272C>T	CAA18877.1:p.Gln758*
CU329672	1642507^1642508	Insertion	–>A	44	–	–

Table 5. Variants located in the S. pombe ird11 strain and not in the 927h⁻ wild type

Chromosome	Location	Type	Change	Read Depth	Coding Region Change	Amino Acid Change
CU329670	660057..660058	Deletion	GG>–	109	–	–
CU329670	2200711	SNV	G>A	51	CAB66170.1:c.1537C>T	CAB66170.1:p.Arg513*
CU329670	3584035	SNV	G>A	128	CAB16570.1:c.68-40C>T	–
CU329670	4080076	SNV	G>A	255	CAB10128.2:c.875C>T	CAB10128.2:p.Ser292Leu
CU329670	4413184	SNV	G>T	102	CAB66469.1:c.736C>A	–
CU329670	5070354	SNV	G>A	139	CAB60232.1:c.659A>G	CAB60232.1:p.Tyr220Cys
CU329671	1598043	SNV	C>A	25	–	–
CU329671	3831507^3831508	Insertion	–>A	55	CAB76056.1:c.2986_2987insT	CAB76056.1:p.Tyr996fs
CU329672	480848	SNV	C>T	83	CAA18286.1:c.999C>T	–
CU329672	525233	SNV	G>A	95	–	–
CU329672	1174775	SNV	G>A	115	CAB60677.1:c.1286G>A	CAB60677.1:p.Ser429Asn

Table 6. Candidate DNA mutations responsible for the formation of S. pombe ird5 and ird11 mutants

		Type	Region	Location	Chromosome
Ird5h⁻/972h⁻	SPAC12G12.03,cip2	SNV (C>T)	3'UTR	342539	I
	SPNCRNA.1063	SNV (G>A)	–	5267920	I
	SPBC1709.17, met7	SNV (C>T)	ORF	1133028	II
	SPBC23E6.09, *ssn6*	SNV (C>T)	ORF	3865776	II
	1642507/8 Chr III	Insertion	–	1642507/8	III
Ird11h⁻/972h⁻	SPAC1D4.11c	Deletion	–	660057/8	I
	SPAC17A2.13c, *rad25*	SNV (C>T)	Intron	3584035	I
	SPBTRNAASN.01	SNV (C>A)	–	1598043	II
	SPCC4B3.03c, *mam301*	SNV (G>A)	ORF	1174775	III

McCartney et al., 2014), *Kluyveromyces marxianus* (Nguyen et al., 2015), and *S. pombe* (Mehta et al., 1998) in order to understand glucose transport and increased ethanol fermentation from biomass. Mikumo et al. (2015) isolated *S. cerevisiae* strains resistant to glucose repression from cherry fruits using 2-DOG in order to develop yeasts that can use different carbon sources. McCartney et al. (2014) aimed to identify deletion mutants resistant to glucose repression obtained from the *S. cerevisiae* Genome Deletion Project by using 2-DOG, and they performed oligonucleotide-directed mutagenesis. In their study, they reported that a mutation in the *snf1* gene causes 2-DOG hypersensitivity, while another activating mutation in the *snf1* gene causes 2-DOG resistance (McCartney et al., 2014). The orthologue of the *snf1* gene in *S. cerevisiae* is the *ssp2* gene in *S. pombe* (Matsuzawa et al., 2012), and in our present study, we did not observe any alteration in the *ssp2* gene according to the results of whole-genome sequencing.

Since *S. pombe ird* mutants carry a recessive mutation (Kig et al., 2005), at the selection of these transformants, disappearance of resistance to glucose suppression in backward mutations (glucose repression) and formation of resistance to glucose suppression in forward mutations (glucose de-repression) were investigated.

In this study, in order to determine the mutation leading to glucose suppression and oxidative stress resistance phenotype in *S. pombe ird5* and *ird11* mutant strains, firstly, wild alleles (Ssn6T and Mam301T) and the mutant variant (Ssn6D) of genes carrying mutations in protein-coding regions (*ssn6* and *mam301*) detected in sequence analysis were cloned into the yeast expression vector (pSGP572) (Fig. 2). The obtained pSGP572-Ssn6T, pSGP572-Ssn6D and pSGP572-Mam301T plasmids were confirmed by sequencing, and these plasmids were transformed to the ird5u and *S. pombe mam301Δ* strains, respectively and their backward mutations were performed. After selection, pSGP572-ssn6T transformants continued to grow on 2-DOG-containing media. After transformation of the pSGP572-mam301T plasmid into the *mam301Δ* strain, disappearance of 2-DOG resistance was observed in six of 101 transformant colonies (Fig. 3). On the other hand, pJET1.2 cloning vector recombinants (*cip2*, *met7*, *rad25*, SPNCRNA.1063, *mam301*, *ssn6*) and cassettes (SPBTRNAASN.01, SPAC1D4.11c and 1642507/8 Chr III) (Fig. 2) were

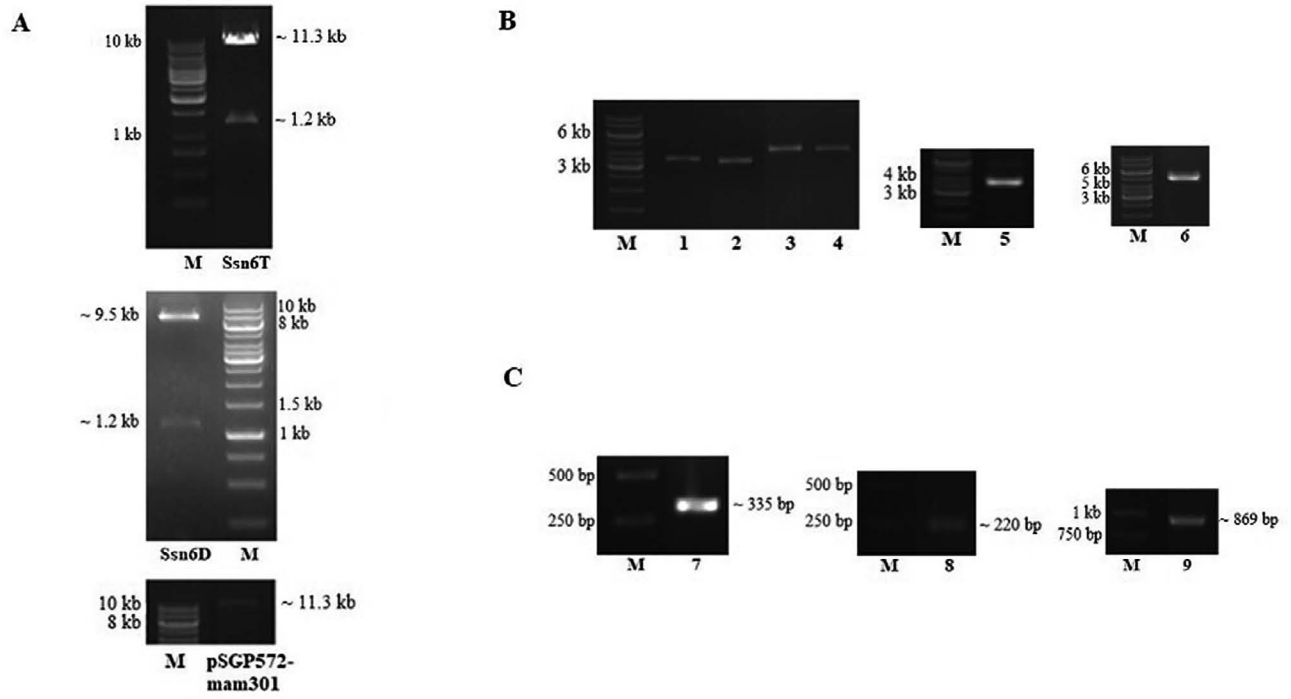

Fig. 2. Agarose gel images of created plasmids and cassettes. **A**. Agarose gel images of pSGP572 plasmids containing Ssn6T/Ssn6D cut with *Eco*RI restriction endonuclease and of pSGP572 plasmid containing *mam301* cut with *Bam*HI restriction endonuclease. (M: ThermoScientific GeneRuler 1 kb DNA Ladder). **B**. Agarose gel images of the pJET1.2/blunt cloning vectors, containing the modified *cip2* (1), *met7* (2), *ssn6* * (4), *rad25* (5), *mam301* (6) gene and SPNCRNA.1063 (3) region, cut with *Xho*I restriction endonuclease. (Expected product size, ~3576 bp for *cip2*; ~3419 bp for *met7*; ~3844 bp for SPNCRNA.1063; ~3638 bp for *ssn6*; ~3865 bp for *rad25*; ~4994 bp for *mam301*). **C**. Agarose gel images of PCR products which belong to SPBTRNAASN.01 (7), 1642507/8 Chr III (8) and SPAC1D4.11c (9) region containing the target mutation.

transformed into the wild type, and thus their forward mutations were performed. Then, these transformant cells were selected in YEA and YEA with 2-DOG media. However, no effective response to glucose suppression was observed (Fig. 4).

The continuing resistance to 2-DOG in the ird5u transformants that include pSGP572-Ssn6T and pSGP572-Ssn6D plasmids checked by sequencing indicated that the *ssn6* gene was not responsible for the formation of the *ird5* phenotype. After it was confirmed that the *S. pombe mam301Δ* strain is resistant to glucose suppression (Fig. 3), carrying out of backward mutation in this strain by using pSGP572-mam301T plasmid suggested that the *mam301* gene may be responsible for the formation of the *S. pombe ird11* phenotype.

It was determined, according to the NCBI database, that the point mutation (change of guanine to adenine (G>A) in 1285th nucleotide) detected in the *mam301* target gene is responsible for the change of the 429th serine amino acid to the asparagine amino acid in the Mam301p polypeptide. The fact that the S429 residue in Mam301p plays a role in the O-phospho-L-serine modification (Kettenbach et al., 2015) made this mutation even more important.

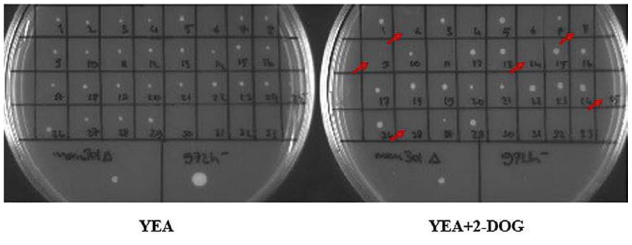

Fig. 3. Growth of selected transformants in YEA and YEA with 2-DOG media, after transformation of pSGP572-mam301T into *S. pombe mam301Δ* cells.

Conclusion

In mutants obtained with random mutations using chemicals, the identification of mutations (intergenic regions, etc.) is quite difficult except for the whole-genome sequencing technique (Forsburg, 2001; Schneeberger, 2014). EMS-induced mutagenesis and mutations provide important opportunities to investigate gene functions and the effect of mutations. In humans and in model organisms, the genome function, regulation, and organization were elucidated by mutation analysis studies.

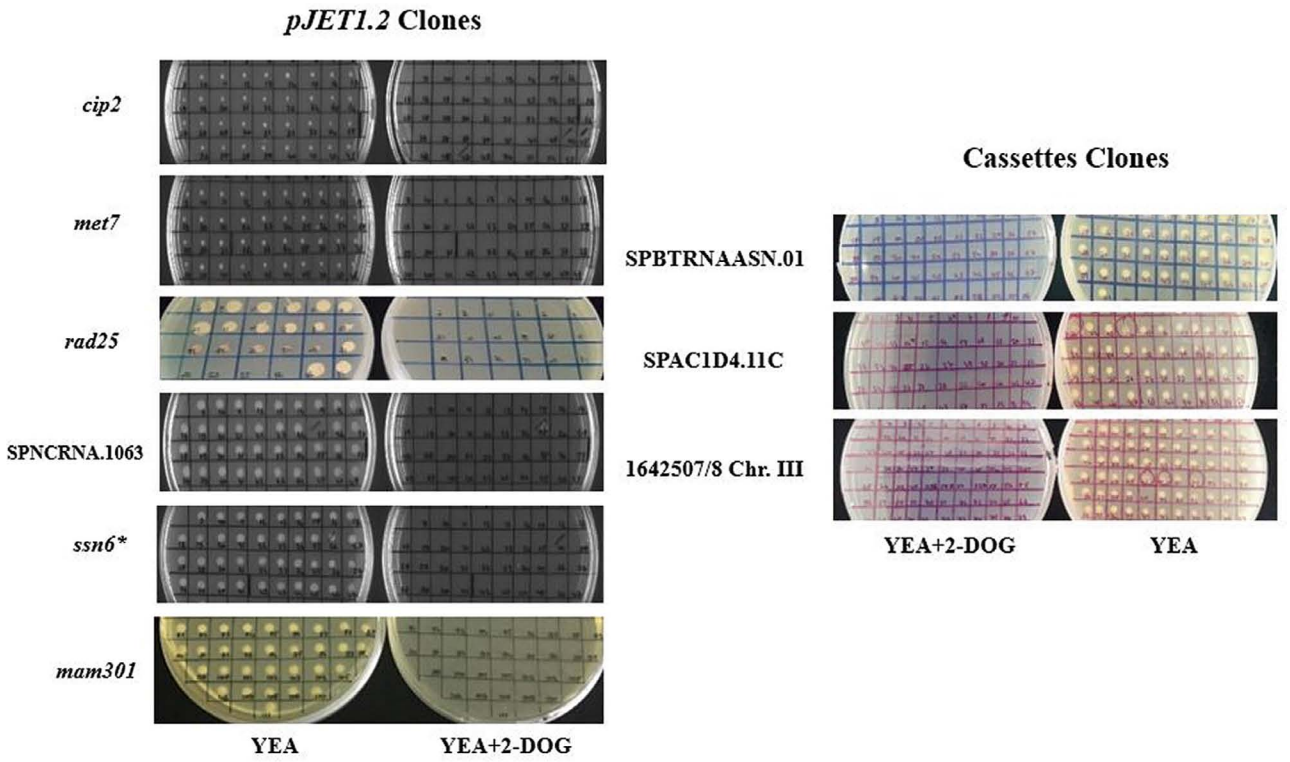

Fig. 4. Growth of transformant cells that included pJET1.2/blunt cloning vector recombinants (*cip2, met7, rad25, mam301, ssn6** genes, SPNCRNA.1063) and cassettes (SPBTRNAASN.01, SPAC1D4.11c and 1642507/8 Chr III) on YEA and YEA with 2-DOG media.

In these studies, EMS-induced mutations were investigated by sequencing (Blumenstiel et al., 2009; Henry et al., 2014; Siddique et al., 2020; Yan et al., 2021). In our study, *S. pombe ird* mutants were randomly obtained using a mutagenic chemical (EMS), and selected using media containing sucrose and 2-deoxyglucose (2-DOG), which is a glucose analogue, as a carbon source (Kig et al. 2005).

The possibility that *mam301* is responsible for the *ird11* phenotype requires a detailed structural and functional analysis of *mam301*. On the other hand, this mutation must be confirmed by forward mutation. In this study, the results of *mam301* gene analysis are not a sufficient finding to reach a definitive conclusion. In chemical-induced mutagenesis studies, more than one gene are affected, unfortunately. It was determined in our previous studies that the *ird5* and *ird11* mutants we used in this study were resistant to oxidative stress and glucose suppression. According to our study, it seems that the cause of resistance may originate from more than one region. When the nine different regions we selected were examined one by one, we could not get an effective result. Additionally, 20 candidate mutations that we revealed as a result of whole-genome sequencing should be investigated in detail with more effective methods such as CRISPR. Elucidation of the relationship between the glucose signalling pathways and oxidative stress response through candidate mutations made in this study can shed light on both aging and stress-related diseases in humans as well as contribute to basic research.

Competing interests

The authors declare no competing interests.

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