Identification of Schizosaccharomyces Pombe Ird Mutants Resistant to Glucose Suppression and Oxidative Stress

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Identification of *Schizosaccharomyces pombe ird* mutants resistant to glucose suppression and oxidative stress

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Abstract

Background: Glucose both is the favorite carbon and energy source and acts as a hormone that plays a regulated role in many biological processes. Calorie restriction extended lifespan in many organisms, including *Schizosaccharomyces pombe,* while uptake of high glucose led to undesired results, such as diabetes and aging.

Methods and Results: 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. Candidate 9 mutations, which are thought to be responsible for *ird5* and *ird11* mutant phenotypes, were investigated via forward and reverse mutations by using various cloning techniques.

Conclusion: The results of this study contribute to the basic sciences by showing the relationship between glucose sensing/signaling and oxidative stress response components.

Keywords: Glucose suppression, oxidative stress, signaling, *Schizosaccharomyces pombe*

Introduction

Glucose plays an important role in the regulation of the expression of genes involved in glucose sensing and signal transduction, transport, catabolism, use of alternative carbon sources, spore formation, and stress response pathways [1-5]. In *Schizosaccharomyces pombe*, unlike *Saccharomyces cerevisiae*, 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) [6,7]. 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) [8].

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 [9] or a defense mechanism against the increased accumulation of reactive oxygen species (ROS) arising from aerobic respiration [10].

In our previous studies, *S. pombe ird* mutants were obtained to be mutagenized with ethyl methanesulfonate (EMS), and selected on the basis of resistance to repression in the presence of a non-metabolizable glucose analog, 2-deoxy,D-glucose (2-DOG). It was indicated that these mutants were two different complementation groups according to random spore analysis. Initial characterization of these mutants, there appeared to be a correlation between low glucose consumption and low glucose repression in *ird11* mutant, while a higher level of glucose repression of *ird5*, although the glucose consumption rate of this mutant is low [11]. The following studies, revealed that *S. pombe ird11* was always resistant to oxidative stress, irrespective of calorie restriction, while *S. pombe ird5* mutant exhibit an adaptive stress response due to glucose deficiency [12,13]. In another study using *ird* mutants as models, lifespan extension seemed to be related to glucose sensing/signaling rather than oxidative stress response and trehalose accumulation [14].

These studies towards aim to clarify the possible relationships between glucose signaling, 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 step of the glucose sensing and signaling pathways in *S. pombe*.

S. pombe genome can be easily altered by homologous recombination, and it can also perform non-homologous recombination. Integration into an auxotrophic locus can be achieved with any plasmid that does not contain ars and contains the appropriate sequence. For example, the plasmid containing a related construct can be integrated into leu1-32 [15]. Whole gene disruption or displacement (knock-out / knock-in) requires additional planning. High integration efficiency occurs using homologous sequences more than 300 bp [16]. Homology less than 100 bp may also be used [17], 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.

The next-generation sequencing technology uses a fundamentally different approach from classical Sanger chain-termination methods. It is sequenced by synthesis. This technique is based on the detection of DNA polymerase activity through a chemiluminescent enzyme. The sequencing reaction is cDNA based performed on single-stranded DNA (ssDNA). First, double-stranded DNA is divided into pieces of 300-800 bp (nebulization), and then, adapter DNA fragments are added. Clonal expansion of the fixed DNA fragments is performed. In this way, large genomes can be sequenced quickly, cheaply and with high accuracy and analyzed [18,19].

In this study, to determine the mutation leading to glucose repression and oxidative stress resistance phenotype in *S. pombe ird5* and *ird11* mutant strains, first all genome analyses were performed with next generation sequencing technology (MiSeq System Illumina). Next, candidate mutation(s) in protein coding and non-coding regions were confirmed by forward and backward mutations using various cloning techniques.

Material and Methods

Yeast Strains and Media

In this study, *ird* mutant strains (*ird5h*$^-$ and *ird11h*$^-$) resistant to glucose suppression, *ura4-D18h*$^+$, *ird5ura4-D18h*$^-$, *mam301Δ* (*ade6-M210 ura4-D18 leu1-32*, SPCC4B3.03c:kanMX4 (from Bioneer)) and wild-type (*972h*$^-$) *S. pombe Lindner liquefaciens* were used. *Escherichia coli* DH5α (dlacZ Delta M15 Delta(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1) was used at the reproduction of the plasmids. Standard yeast extract-enriched media [0.5% yeast extract, 3% glucose and 2% agar] and selective media [0.5% yeast extract, 3% sucrose and 400 µg/ml 2-deoxy-D-glucose (2-DOG)] were used for *S. pombe ird* mutants [20,21].

DNA Isolation and Whole Genome Sequencing

S. pombe genomic DNA isolation was performed according to the method developed by Bähler et al. (1998) [17]. 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 (3000 rpm/min).

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 homogenized by a dismembrator for 4 minutes at 3000 rpm. The mixture was centrifuged, and the supernatant was removed and centrifuged again (13,000 rpm) 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.

Whole genome sequencing of *S. pombe* strains was carried out on the “MiSeq System Illumina” device by service procurement. To determine the differences between mutant strains and wild-type strains, whole genome sequencing of each DNA sample was performed by next-generation sequencing system, and then, the mutations that were found on genomes were identified.

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

The obtained data were aligned according to the reference genome, and the *Schizosaccharomyces pombe* genome (GenBank ID: CU329670.1, CU329671.1 and CU329672.1) in the literature was used as the reference genome during the alignment process [22]. Alignments to three different chromosomes in the reference genome were performed using the "BWA mem v.0.7.12" program. After the alignment, data were filtered according to the quality control with the 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, completion of annotation process for the obtained variation list, filtration of variation lists according to strand bias by taking upper limit (20%), and elimination of unreliable variations (<80%) according to the detected variation percentage were performed. 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.

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, wild allells (Ssn6T and Mam301T) and mutant variants (Ssn6D) of the target genes *(ssn6* and *mam301*) were cloned into the yeast expression vector (*pSGP572*). The resulting pSGP572-

Ssn6T, pSGP572-Ssn6D and pSGP572-Mam301T recombinants were transferred to *ird5ura4D18* and *S. pombe mam301Δ* strains, respectively, to perform reverse mutations. On the other hand, the forward mutation of *S. pombe* wild type (*972h*$^{-}$) was performed by transferring both pJET1.2 cloning vector which contains the mutant variants of "*cip2*, *met7, rad25*, SPNCRNA.1063, *mam301, ssn6*" and cassettes including mutant variants of "SPBTRNAASN.01, SPNCRNA.671 and 1642507/8 nucleotide in Chr III".

Plasmids

For the cloning and expression of the *ssn6* and *mam301* genes, the expression vector *pSGP572*, commercially available from NBRP, a research center in Japan, designed by Susan L. Forsburg, was used. This vector includes regulatable promoter of the *nmt1* gene whose expression by thiamine can be suppressed (23). Additionally *pSGP572* involves *ura4+* gene for *S. pombe* and amp^{R} gene for *E. coli* as marker gene. Thermo Scientific CloneJET PCR Cloning Kit was used to clone some targeted protein coding and non-coding regions (*Cip2*, *met7*, *rad25*, SPNCRNA.1063, *mam301*, *ssn6**).

Obtaining of double mutants in S. pombe

To obtain the double mutants of *S. pombe ird5h*$^{-}$ and *ura4h*$^{+}$, the cells of each strain were crossed on the SPA media and incubated for 3 days at 25ºC, 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 mating-type of double mutant cells were determined by crossing with the wild type cells (20).

Formation and transformation of plasmids and cassettes

The polymerase chain reaction (PCR) was carried out with the Thermo Scientific Phusion High-Fidelity DNA Polymerase enzyme by using *S.pombe 972h*$^{-}$ wild type genome DNA as template. Appropriate primers were designed by using the primer design tool "Integrated DNA Technologies" (https://eu.idtdna.com/Primerquest/Home/Index) for the reconstruction of candidate DNA mutations responsible for the formation of *S. pombe ird5* and *ird11* phenotypes (Table 1). The primers given in Table 1 were used for amplification of target genes or extragenic regions.

Table 1 The primer sequences used in the study and their melting temperatures (Tm ° C)

	Forward primers	Reverse primers	Tm (°C)
ssn6 wild allel	TTTTGTCGACATGCCCCAATCACAAGTCGCT	AAGGATCCTTAAACTGACACGGTTTCTTT	50
ssn6 mutant variant	TTTTGTCGACATGCCCCAATCACAAGTCGCT	AAGGATCCTTAACCGGTAGCAGAAGGAAG	54
SPBTRNAASN.01	F: ACTCGCATTTGCTGTCAATTT	R: TTGGTTATTGCTCACGGACTG TTAA	55
	-	R1: GCATTATAGGTCGGGTAGCATAG	51
cip2	F: CCCAGCTTGTTCACGTTAGT	R: GCCGGCAAATAGCCAATAGA	47
	F1: ATTATTTTTTTTCGCACTGTTTTTAG TGGATACTTAATGATTTTGTCTAA	R1: TACAGCAGATACGAGATACGTTTAGAC AAAATCATTAAGTATCCACTAAAA	45
III. chr. 1642507/8	F: AAATGCTAAAGGCCGCTAAAG	R: TAATCGCTTTTTTTTGATGTTTTTTGT	42
	-	R1: AGTTGTTGATGCACCATTGAA	43
met7	F: TGGCAAACCTATCAGTCAAGAG	R: ACCAATCATATCCGGCGTTAAT	46
	F1: GCCAATGTATTTTCGATTCTTGAC	R1: AGTGTCAAGAATCGAAAATACAT	45
rad25	F: CGATTGAACTGCCTTGATTGTC	R: GCGAAGAAGCTGCATGATTAAA	49
	F1: TTCATCTCTCTTTTTTGTTTTTGC	R1: AACAAAAAAGAGAGATGAAAGAA	49
SPNCRNA.671	F: TGTAGGGTGCAACAGTAAAGAG	R: GATGGTGATTGTTGGGTTGTTC	48
	F1: AATGGGGGGGGTAAAACAACAAAGCA	R1: CCCCCCCCATTGGAATTACATTCTGT	48
SPNCRNA.1063	F: CCAAACAATCCCTATCCTCTTCT	R: CCAGATTCCCGTACCTTGATATT	50
	F1: TTAAGGTTGTTTACTGAACTATCCG	R1: CAGTAAACAACCTTAATTGTGTCG	41
mam301	F: ACGCGTCGACATGTCCCTATTGAGAATT	R: CGGGATCCTTTCTTGCTTTTACCTTT	47
	F1: TACAAGAAAAGCATGCCGAGAG	R1: TCTTGTAAATCCAAAACAGCTG	44
*ssn6** mutant variant	F: GCTGTGTATCGTGATGGTAGAA	R: GAGGAGACTTGGTAGACGAAATAG	47
	F1: TACCGGTTAATCAGGCGTCGTGCAA	R1: CGCCTGATTAACCGGTAGCAGAA	50

Recognition sequences of *Sal*I and *BamH*I restriction endonucleases were added to each of the primers designed to clone the *ssn6* and *mam301* genes into plasmid *pSGP572*, unlike other sequences. Using three separate techniques, it was aimed transformation of target sequences into cells (Figure 1). The lithium acetate method of Gregan *et al* (2006) [24] was used for the transformation of the prepared plasmids and cassettes into *S. pombe* cells.

Figure 1 Different cloning techniques used in this study. A. After the 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. B. Using primers containing altered nucleotides, the target region is amplified by PCR and transformation into the cell is performed. 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 altered nucleotides and allowed to ligation. Then plasmid containing altered gene was transformed into the cell.

PCR products of wild alleles and mutant variants of the *ssn6* and *mam301* genes and plasmid *pSGP572* were cut with appropriate restriction endonucleases and then ligated by T4 ligase (Invitrogen). pSGP572-ssn6D and pSGP572-ssn6T recombinants were transformed into *S. pombe ird5ura4h*$^-$ mutants; and pSGP572-mam301 recombinant was transformed into the *S. pombe mam301Δ* strain. After transformation, *ird5ura4h*$^-$ transformants were selected on MMA, MMA with uracil and MMA with 2-DOG media. On the other hand *mam301Δ* transformants were selected on MMA with adenine and leucine and YEA containing 2-DOG media to determine glucose suppression.

Sequences of SPBTRNAASN.01, SPNCRNA.671 and sequences including the changes in nucleotide 1642507/8 on chromosome 3 were amplified by PCR and then this constructs were directly transformed to the *S. pombe 972h*$^{-}$ wild strain in linear form (cassette structure). Transformants were selected on YEA and YEA with 2-DOG media.

Cip2, *met7*, *rad25*, SPNCRNA.1063, *mam301*, *ssn6** genes and extragenic regions were cloned using the "Thermo Scientific CloneJET PCR Cloning kit" according to the manufacturer's instructions. Vectors containing the mutant alleles of the genes were formed by amplifying vectors containing the target genes with second set of primers (F1,R1 primers, Table 1) prepared for each gene. After these amplifying reactions, ligations of vectors were performed with T4 ligase and these pJET1.2 recombinants were transformed into *S. pombe 972h*$^{-}$ wild type. Transformants were selected on YEA and YEA with 2-DOG media.

Results

After genomic DNA isolation from *S. pombe* wild type and *ird* mutants, genomic DNA samples were checked by agarose gel electrophoresis. These DNA samples were used for next-generation sequencing and analysis. After sequencing, 12.3 M reads and 6 Gb of data were obtained as 2×150 bp reads for 3 samples. The number of readings and amount of data obtained for each sample are given in Table 2. The raw data generated from the reads were obtained in *fastq format, and quality control of Fastq data was performed by the "FastQC" program.

Table 2 Reading counts, data and mean reading depths of samples

Samples	Reading count (2×150)	Data	Mean Reading Depth
972h^{-}	3213644	1.649 GB	37
ird5	2835568	1.455 GB	31
ird11	5482099	2.813 GB	54

After alignment of the *Schizosaccharomyces pombe* genome (GenBank ID: CU329670.1, CU329671.1 and CU329672.1) to the three different chromosomes, nucleotide and amino acid changes in the coding region, localizations, types, changes, and reading depths of the variants that showed differences between mutant strains (*ird5, ird11*) and wild type (*927h*$^{-}$) are indicated (Table 3-4). We selected 10 candidate DNA mutations regions in this study and the location and type of mutations in the targeted protein coding and non-coding regions are given in Table 5.

Table 3 Variants located in the *S. pombe ird5* strain and not in the *927h*$^{-}$ wild type

Chromosome	Location	Type	Change	Reading 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.50	-

					7G>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 4 Variants located in the *S. pombe ird11* strain and not in the *927h*$^-$ wild type

Chromosome	Location	Type	Change	Reading 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 5 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
	CU329672	insertion	-	1642507/8	III
Ird11h-/972h-	CU329670, SPCRNA.671	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

Target Sequences and Plasmids Containing Target Sequences

pSGP572 plasmids including wild alleles and mutant variants of the *ssn6* and *mam301* genes, pJET1.2/blunt cloning vector including genes and extragenic regions (*Cip2* (602 bp), *met7*(445 bp), *rad25*(891 bp), SPNCRNA.1063(870 bp), *mam301*(2020 bp), *ssn6**(664 bp)) and cassettes were controlled (Figure 2). pSGP572-ssn6D and pSGP572-ssn6T plasmids were confirmed by sequencing.

Figure 2 Agarose gel images of created plasmids and cassettes. A. Agarose gel images of *pSGP572* plasmids containing Ssn6T/Ssn6D cut with *EcoRI* restriction endonuclease and of *pSGP572* plasmid containing *mam301* cut with *BamHI* restriction endonuclease. (M: ThermoScientific GeneRuler 1 kb DNA Ladder) B. Agarose gel images of the vectors, containing the modified *cip2* (1), *met7* (2), *ssn6* * (4), *rad25* (5), *mam301* (6) gene and SPNCRNA.1063 (3) region, cut with *XhoI* 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 gene and 1642507/8 Chr III region containing the target mutation.

Transformation of Plasmids and Cassettes

pSGP572-ssn6D and pSGP572-ssn6T plasmids were transformed into *S. pombe ird5ura4h*$^{-}$ strain. After selection, pSGP572-ssn6T transformants continued to grow on 2-DOG-containing media. After transformation of pSGP572-mam301 plasmid into *mam301Δ* strain, disappearance of 2-DOG resistance was observed in 2 of 101 transformant colonies (Figure 3).

Figure 3 Growth of selected transformants in YEA and YEA with 2-DOG media respectively, after transformation of the pSGP572-mam301 into *S. pombe mam301Δ* cells.

pJET1.2/blunt cloning vector recombinants (*Cip2*, *met7*, *rad25*, SPNCRNA.1063, *mam301*, *ssn6**) and cassettes (SPBTRNAASN.01, SPNCRNA.671 and nucleotides 1642507/8 on Chr 3) were transformed into wild type cells and selected in YEA and YEA with 2-DOG media. However, no effective response to glucose suppression was observed.

Discussion

In mutant strains obtained with random mutations using chemicals, the identification of mutations (intergenic regions, etc.) is quite difficult except for whole genome sequencing [25,26]. For this reason, the *ird* mutants needed to be identified by whole genome sequencing.

S. pombe ird mutants were randomly obtained using a mutagenic chemical, ethyl methyl sulfonate (EMS), and selected using media containing sucrose and 2-deoxyglucose (2-DOG), which is a glucose analog, as a carbon source [11].

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 was detected in the *ird5* mutant. 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 *ird11* mutant. Similar differences in *ird5* and *ird11* were detected at different alteration depths. These alterations may be due to the formation of the *ird* mutants.

A total of 20 different mutations were identified in *ird* mutant genomes involved in two different complementation groups of *S. pombe* [11]. The locations of these mutations were interrogated in the *S. pombe* genome using the National Center for Biotechnology Information (NCBI) database. Of these, 12 were identified in protein-coding regions (SPAC16E8.15, SPAC29E6.06c, SPAC1006.02, SPAP8A3.07c, SPAC869.06c, SPBC1709.17, SPBC23E6.09, SPAC1783.07c, SPAC19G12.16c, SPBC21C3.20c, SPCC364.04c, SPCC4B3.03c), 5 were found in regulatory regions of a gene (SPAC12G12.03, SPAC17A2.13c,

SPNCRNA.1063-SPNCRNA.1064, SPCC663.18, SPBTRNAASN.01), and 3 were detected in intergenic regions (CU329670, CU329671, CU329672) in the genome. Most of these mutations were observed as single nucleotide variations. These alterations were consistent with the applied chemical mutagen. 10 of the 17 genes that contain the protein coding region and the altered regulatory regions are orthologous with human. We selected 9 different protein coding and non-coding regions (SPAC19G12.16c (*cip2*), SPNCRNA.1063, SPBC1709.17 (*met7*), SPBC23E6.09 (*ssn6),* 1642507/8 on Chr. III, SPCRNA.671, SPAC17A2.13c (*rad25),* SPBTRNAASN.01, SPCC4B3.03c (*mam301)*).

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, mutant variants (Ssn6D) and wild alleles (Ssn6T and Mam301T) of genes carrying mutations in protein coding regions (*ssn6* and *mam301*) detected in sequence analysis were cloned into the yeast expression vector (*pSGP572*). Obtained pSGP572-Ssn6T, pSGP572-Ssn6D and pSGP572-Mam301T recombinants were transformed to the *ird5ura4D18* and *S. pombe mam301Δ* strains, respectively and their reverse mutations were performed. On the other hand pJET1.2/blunt cloning vector including mutant variants of targeted intragenic and extragenic regions (*cip2*, *met7*, *rad25*, SPNCRNA.1063, *mam301*, *ssn6*) and cassettes including mutant variants of other targeted regions (SPBTRNAASN.01, SPNCRNA.671 and nucleotides 1642507/8 on Chr 3) were transformed to *S. pombe* wild type and their forward mutations were performed. However, the desired results could not be obtained.

Since *S. pombe ird* mutants carry a recessive mutation [11], at the selection of these transformants, disappearance of resistance to glucose suppression in reverse mutations (glucose repression) and formation of resistance to glucose suppression in forward mutations (glucose de-repression) were investigated.

The continuousness of resistance to 2-DOG in the *ird5ura4h*$^{-}$ transformants which include Ssn6T or Ssn6D-containing plasmids controlled 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 (Figure 3), carrying out of reverse mutation in this strain by using pSGP572-mam301 plasmid suggested that the *mam301* gene may be responsible for the formation of the *S. pombe ird11* phenotype.

It was determined in the NCBI database (http://www.ncbi.nlm.nih.gov/nuccore/159883886?report= graph) [27] that the point mutation (change of guanine to adenine (G>A) in 1285. nucleotide) detected in the target gene *mam301*, is responsible for the change of the 429. serine amino acid to the asparagine amino acid in the Mam301p polypeptide. The fact that the residue S429 in Mam301p plays role in the O-phospho-L-serine modification [28] made this mutation even more important.

The possibility that *mam301* is responsible for the phenotype *ird11* 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 are not a sufficient finding to reach a definitive conclusion. Consequently, *mam301* gene might be responsible for *S. pombe ird11* phenotype, while we could not determine any mutations related to *S. pombe ird5* phenotype among our target regions. 20 candidate mutations that we revealed as a result of whole genome sequencing should be investigated in detail with more modern and effective methods such as CRISPR. It has been foreseen that demonstration of regulation of the relationship between glucose signaling pathways and oxidative stress response through candidate mutations from this study can shed light on the studies about both aging and stress-related diseases in human as well as it contributes to basic science.

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Declarations

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Conflicts of interest

The authors declare no competing interests.

Author's contribution

Bedia Palabiyik conceived and designed the experiments. Merve Yilmazer, Beste Bayrak, Burcu Kartal and Semian Karaer Uzuner performed experiments. Bedia Palabiyik, Merve Yilmazer, discussed the results and wrote the article.

Figures

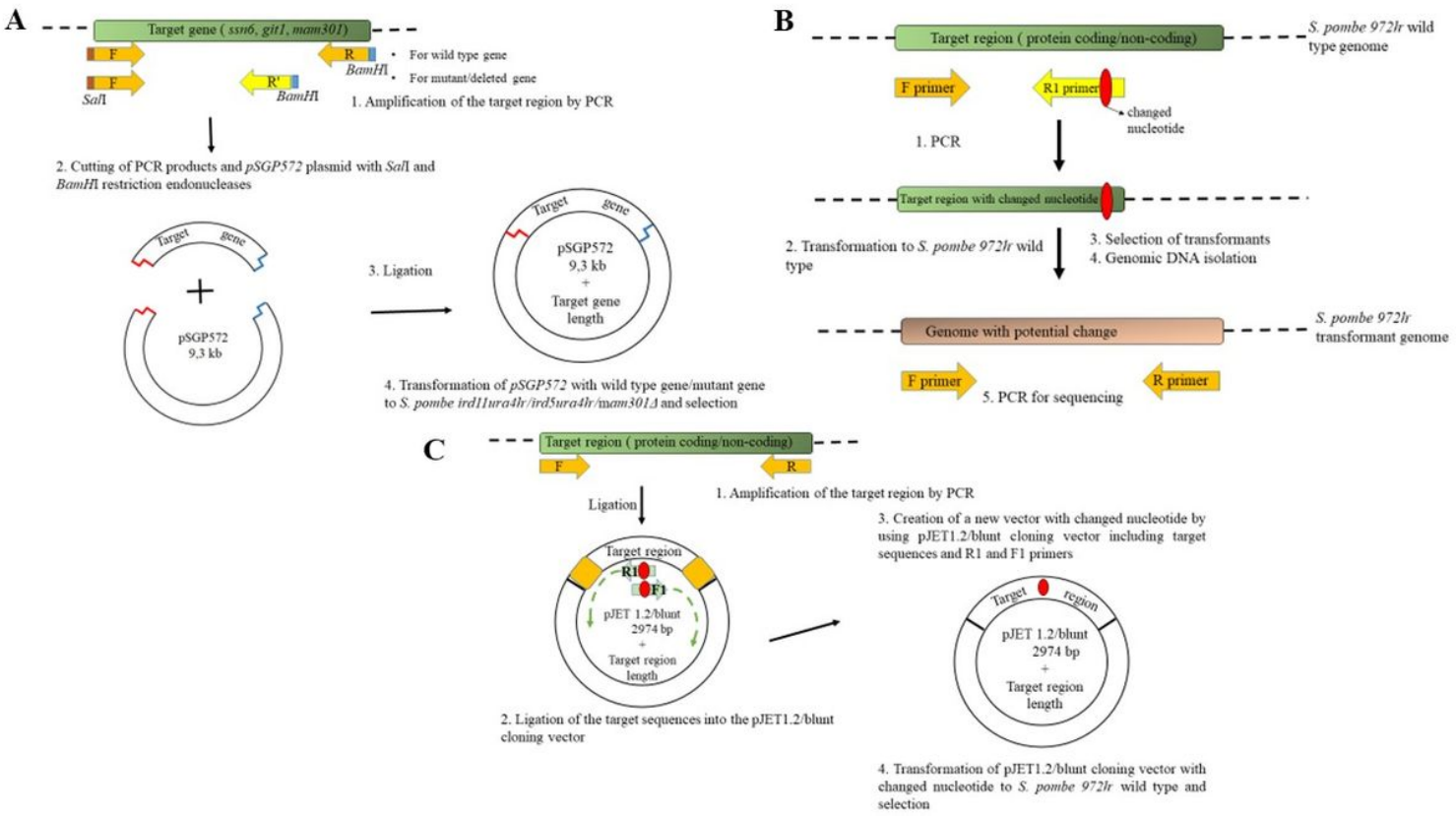

Figure 1

Different cloning techniques used in this study. A. After the 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. B. Using primers containing altered nucleotides, the target region is amplified by PCR and transformation into the cell is performed. 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 altered nucleotides and allowed to ligation. Then plasmid containing altered gene was transformed into the cell.

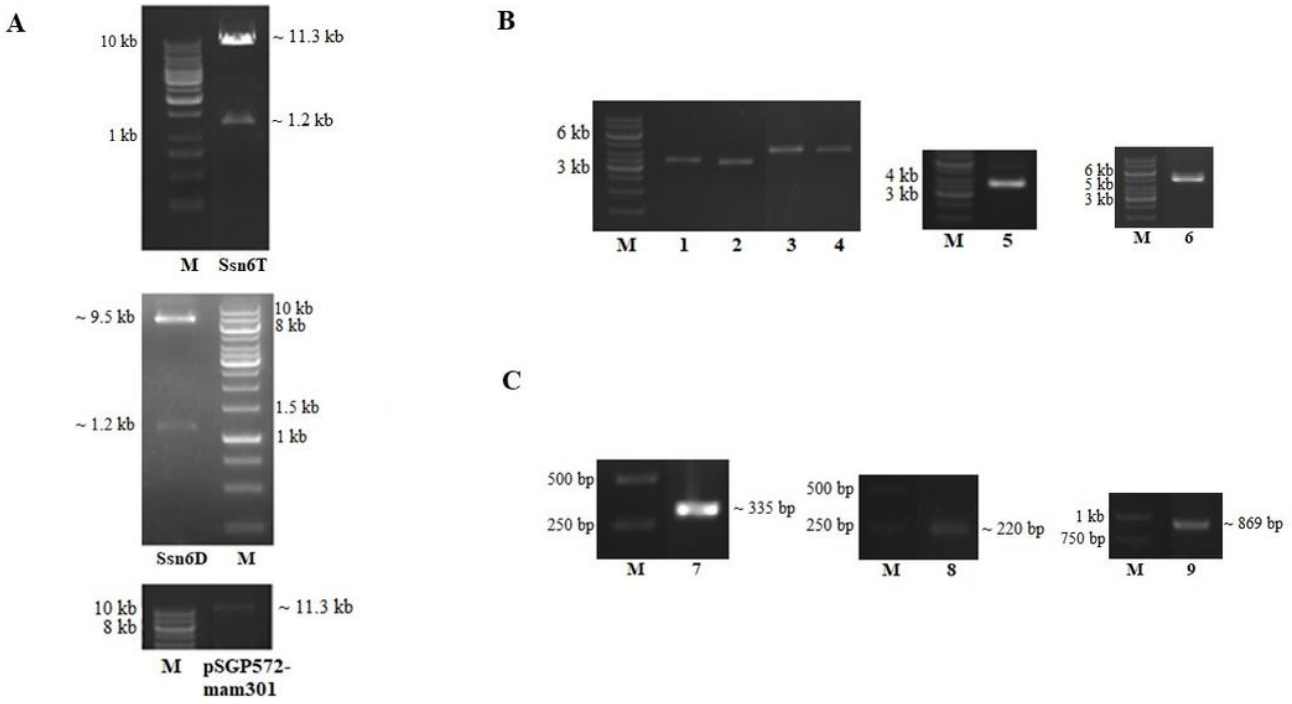

Figure 2

Agarose gel images of created plasmids and cassettes. A. Agarose gel images of pSGP572 plasmids containing Ssn6T/Ssn6D cut with EcoRI restriction endonuclease and of pSGP572 plasmid containing mam301 cut with BamHI restriction endonuclease. (M: ThermoScientific GeneRuler 1 kb DNA Ladder) B. Agarose gel images of the vectors, containing the modified cip2 (1), met7 (2), ssn6 * (4), rad25 (5), mam301 (6) gene and SPNCRNA.1063 (3) region, cut with XhoI 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 gene and 1642507/8 Chr III region containing the target mutation.

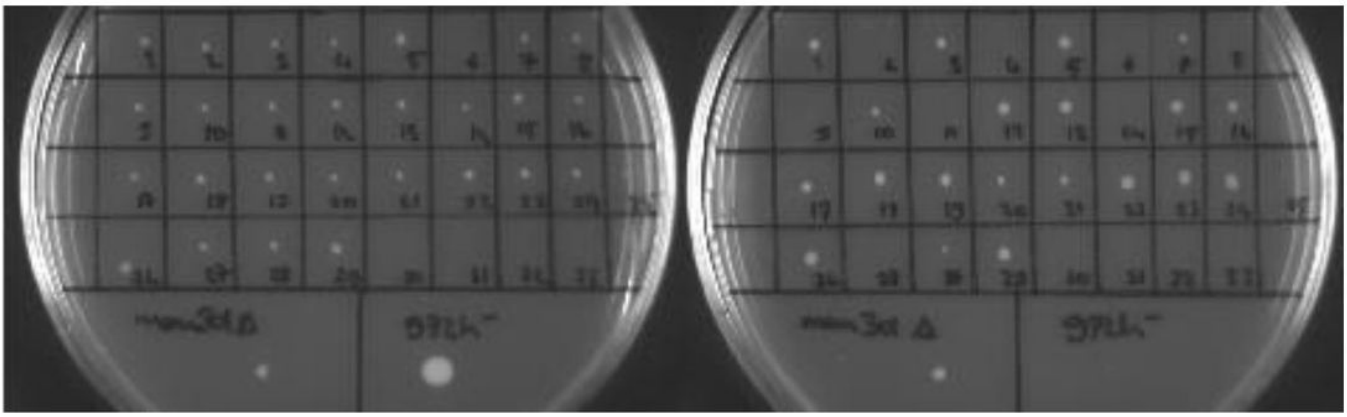

Figure 3

Growth of selected transformants in YEA and YEA with 2-DOG media respectively, after transformation of the pSGP572-mam301 into S. pombe mam301Δ cells.