Title

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Glutamic acid and Glutamyl tRNA synthetases induce sexual differentiation of *Schizosaccharomyces pombe*

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Running title: Glutamic acid and GluRS in fission yeast

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Abstract

The *moc3* gene has been screened out as an inducer of sexual differentiation in *Schizosaccharomyces pombe*. We isolated a novel gene named *ers2* encoding mitochondrial glutamyl tRNA synthetase (mGluRS) as a Moc3 interacting element by yeast two hybrid system. Cytoplasmic glutamyl tRNA synthetase (cGluRS) also interacted with Moc3 in a yeast two hybrid system. Disruption of either *ers1* (cGluRS) or *ers2* (mGluRS) indicated that these genes are both essential for cell growth of *S. pombe*. Interestingly, *ers2* severely affected cell growth and decreased viability but induced sexual differentiation of *S. pombe* when it was over expressed. Over expression of *ers1* also stimulated sexual differentiation of *S. pombe*. These observations lead us to test the effect of various amino acids on sexual differentiation. We found that glutamic acid as well as other amino acids such as tryptophan, methionine and threonine efficiently induced sexual differentiation of *S. pombe*. Our findings suggest a new regulatory mechanism where glutamic acid and its tRNA synthetase involved in sexual differentiation of fission yeast.

Key words: *Schizosaccharomyces pombe*; sexual differentiation; glutamic acid; GluRS

Introduction

Fission yeast *Schizosaccharomyces pombe* cells arrested at the G1 phase when nutrient such as nitrogen or carbon source is depleted. Then the opposite mating type cells, h^- and h^+ initiate conjugation and subsequent zygotes form four ascospores.[1,2] Sexual differentiation of *S. pombe* that precedes meiosis is regulated mainly by three pathways namely the cAMP pathway, the stress responsive pathway and the pheromone signaling pathway.[2,3] Stress-responsive and the pheromone signaling pathway positively regulate the transcription of *ste11*, which encodes a key regulator of sexual differentiation.[3-5] Meanwhile, the nutrient sensing cAMP pathway negatively regulates it through protein kinase A.[6,7] When glucose (or nitrogen) is abundant a heterotrimeric type guanine nucleotide-binding protein (Gpa2) becomes activated,[7,8] this subsequently activates adenylyl cyclase (Cyr1),[8] to generate cAMP from ATP.[9] Cyr1 interacts with its associated protein Cap1, which plays a partly regulatory role of adenylyl cyclase and also interacts with actin and 14-3-3.[10-12] When cAMP is abundant it associates with the regulatory subunit Cgs1 and the catalytic protein kinase Pka1 is released.[13] Pka1 phosphorylates the Zn-finger protein Rst2, which otherwise induces the expression of *ste11*.[14]

The *moc1, moc2, moc3* and *moc4* genes were isolated as to bypass the sterility phenotype of *S. pombe* caused by an elevation of cAMP.[9] Moc1 (Sds23) was also isolated as a potential regulator of M-phase progression,[15] is involved in meiosis,[16] and its two orthologs, budding yeast SDS23 and SDS24 are functionally complementary.[17] Moc2 (Ded1), which is a general translational regulatory factor,[18] and works as an RNA helicase, involves in both sexual differentiation and cell growth.[20] Moc3, a novel Zn finger type protein is involved in sexual

differentiation, ascus formation, and stress response.[20] Moc4 (Zfs1), an mRNA binding protein,[21] involves in sexual differentiation and septum formation.[22] All Moc proteins positively induced sexual differentiation in *S. pombe* in different degrees. Moc1 had the highest, Moc2 had lowest and both Moc3 and Moc4 had intermediate effects.[20] But, there is no evidence till now how Moc1-4 proteins induce sexual differentiation through interaction with other proteins.

In this study, we isolated a novel gene named *ers2* which encodes mitochondrial glutamyl tRNA synthetase (mGluRS) as a Moc3 interacting element. Higher expression of the *ers2* gene resulted in slow growth and induces sexual differentiation of *S. pombe*. We also found the gene named *ers1* encoding cytoplasmic glutamyl tRNA synthetase (cGluRS) stimulated sexual differentiation of *S. pombe*. In addition, glutamic acid was found to induce mating efficiently, suggesting the existence of a regulatory mechanism involved in glutamic acid and its aminoacyl tRNA synthesis.

Materials and methods

Stains, media and genetic manipulation. The *Saccharomyces cerevisiae* and *S. pombe* strains used in this study are listed in Table 1. Standard yeast culture media and genetic manipulations were used, as described previously.[23,24] The *S. cerevisiae* strain was maintained on YPD media composed of 1% yeast extract (Y), 2% bactopeptone (P), 2% dextrose (D) and 2% agar. The *S. pombe* strains were grown in a complete YEA medium (0.5% yeast extract, 2% glucose and 0.0075% adenine) or in the synthetic minimal medium, PM (0.3% potassium hydrogen phthalate, 0.22% sodium phosphate, 0.5% ammonium chloride

2% glucose, vitamins, minerals and salts) with the addition of appropriate auxotrophic supplements (0.0075% adenine, leucine or uracil) when required. LiOAc or electroporation method was used to transform yeast cells.[25,26] *Escherichia coli* DH5α grown in Luria-Bertani (LB) medium (1% polypeptone, 0.5% yeast extract, 1% sodium chloride) hosted all plasmid manipulations and the standard method used for DNA manipulations.[27]

Table1

Plasmid construction. The bait plasmid pGBKT7-moc3 which bears the *moc3* gene with Gal4 DNA binding domain (BD) was constructed as follows. The *moc3* gene was amplified from pMCS33,[20] by PCR using primers moc3-F-SmaI and moc3-R-SalI (Table 2). The PCR product was cloned into the *Sma* I-*Sal* I sites of pGBKT7 that bears Gal4 BD to create pGBKT7-moc3. The sequence of the *moc3* gene was verified from the resulting constructs. Expression of a bait protein (Moc3) fused to the Gal4 DNA binding domain (Gal4 BD) was verified by Western blotting with anti-c-Myc antibody (data not shown).

Similarly, to create pGAD424-moc3, the *moc3* digested fragment was cloned into *Sma*I and *Sal*I sites of pGAD424 that bear the Gal4 activation domain (Gal4 AD). The resulting constructs were also confirmed by restriction digestion and PCR amplification of the respective gene.

The plasmid pGBKT7-mGluRS which bears the *ers2* gene and Gal4 BD was constructed as follows. The *ers2* gene was amplified by PCR from genomic DNA using primers mGluRS-F-EcoRI and mGluRS-R-SalI (Table 2). The amplified fragments were digested by restriction enzymes *EcoR*1 and *Sal*1. The digested fragment was cloned into *EcoR*1 and *Sal*1 sites of pGBKT7 to create pGBKT7-mGluRS. The resulting construct was confirmed by restriction digestion and verified sequence of *ers2*.

Similarly, we constructed pGBKT7-cGluRS where the *ers2* gene was cloned to *Bam*H1 and *Pst*1 sites of two hybrid vector pGBKT7 which bear Gal4 DB.

The plasmids pREP1-mGluRS and pREP81-mGluRS which bear the *ers2* gene under the control of the thiamine repressible promoter, were constructed as follows. The *ers2* gene was amplified by PCR from genomic DNA of *S. pombe* using forward and reverse primers (Table 2). The PCR product was digested with *Sal* I and *Bam* HI and respectively inserted into *LEU1* based pREP1 and pREP81. The vector is differed only in the strength of promoter (the weakest promoter in pREP81).[27,28] The sequence of the *ers2* gene was verified. We also constructed pREP1-cGluRS where the *ers1* gene was cloned to *Bam* HI and *Sma* I sites of pREP1. However, pREP1-lrs1 was constructed where cytoplasmic leucyl tRNA synthetase (*lrs1*) gene was cloned to *Nde* I and *Sal* I sites of pREP1. The resulting plasmids were confirmed through restriction digestion and sequencing. Plasmid manipulation and bacterial transformation were performed using standard techniques.[27]

Table 2

Yeast two hybrid assay. Yeast two-hybrid assay was performed by the method as described earlier.[29] *S. cerevisiae* AH109 cells were transformed with pGBKT7-moc3 and cDNA library by using the Li acetate-polyethylene glycol one-step transformation protocol.[26] Transformants were selected on SC-Leu-Trp-His + 3-AT plate. The 3-AT is a competitive inhibitor of the yeast HIS3 protein (His3p), that was used to inhibit low levels of His3p expression, and thus, to suppress background growth on medium lacking His.[30] The β-galactosidase activity was checked by filter lift assay employing liquid N_2–lysed cells floated on X-Gal

(5-bromo-4-chloro-3-indolyl-D-galactopyranoside)-containing phosphate buffer.

Construction of a GluRS disrupted strain. The *ers2* disrupted strain was constructed by replacing the entire coding region of *ers2* with the *kanMX6* module.[31] The deletion DNA cassette was constructed using the recombinant PCR approach. DNA fragments of 400-500bp and corresponding to the 5` and 3` region of the *ers2* gene were amplified from *S. pombe* by PCR using primers mGluRS (A) and mGluRS (B) and also mGluRS (Y) and mGluRS (Z). Both amplified fragments were used to attach to the end of the *kanMX6* module of pFA6a-kanMX6 by PCR. DNA fragments containing the disrupted *ers2* genes were introduced into h^+/h^+ diploid strain SP826. The transformants were selected with G418 (Sigma Chemical Co.). Proper integration of *ers2* disruption was verified by PCR and Southern blot analysis (data not shown) and the resulting strain *ers2*$^+$/Δ *ers2::kanMX6* was named SKP17. Similarly, by using above techniques with respective primers (Table 2) we constructed *S. pombe* strain SKP19 by replacing one copy of the *ers1* gene to create *ers1*$^+$/Δ *ers1::kanMX6*.

Tetrad analysis. The diploid disruptant SKP17 (*ers2*$^+$/Δ *ers2::kanMX6*) and SKP19 (*ers1*$^+$/Δ*ers1::kanMX6*) were picked up from YE plate, diluted into 300µl dH_20 and plated on PM plates supplemented with appropriate auxotrophic supplements and incubated for 3 days at 30^0C. After growing, colonies were treated with iodine vapor for 7-8 minutes where sporulated colonies were turned into brown color. The brown colonies were picked up and streaked on similar PM plates in appropriate supplement and incubated 4 days at 30^0C. The asci formed

were isolated with a manipulator, and then the plate was incubated several hours at 30^0C. The spores that appeared were dissected through microscope, and the plate was incubated for 4 days at 30^0C for analysis.

Construction of Δ *ers2::kanMX6 (pREP81-mGluRS)*. The haploid strains that had the Δ *ers2::kanMX6* allele complemented by wild type *ers2*$^+$ on plasmids (Δ*ers2/pREP81-mGluRS)* were constructed as follows. Diploid disruptant SKP17 (*ers2*$^+$/Δ*ers2::kanMX6*) cells were transformed with *pREP81-mGluRS*, plated on plates containing PM medium supplemented with appropriate auxotrophic supplements and incubated at 30^0C for 3 days. The visible colonies were streaked onto YE plate. A single colony was picked up from YE plate and suspended into 300µl dH_2O and plated on PMU for 3 days at 30^0C. After growing, colonies were treated with iodine vapor for 7-8 minutes and only spore forming colonies were turned into brown color. The brown colonies were re-streaked onto PMU plates to isolate the sporulated colony and incubated for 4 days at 30^0C. Single colony was suspended into 30% EtOH, vortexed and kept at room temperature for half an hour to kill the vegetative cells and rupture the ascus wall. The ruptured cells were observed under microscope and resulting spores were plated on PMAU plates. Growing haploid clones were streaked onto YES+G418 plates where disrupted Δ *ers2::kanMX6* clones showed resistance to G418. The isolated haploid cells were confirmed through observed the red color after streaked onto YE plate and incubated over night at 30^0C. Thus, isolated cells were haploid Δ *ers2::kanMX6* harboring *pREP81-mGluRS*. The resulting strain was named SKP17-H1.

Mating and sporulation efficiency assay. The mating and sporulation

frequencies were assayed as follows. Cells were grown to the mid-log phase in PM medium with appropriate supplements, washed with nitrogen-free and glucose free PM medium, inoculation in PM medium with various concentrations of nitrogen and glucose, and incubated at 30^0C. After incubation for selected times, 1ml of cell suspension was collected in 1.5 ml eppendorf, sonicated gently for 1 min and the number of zygotes were counted in a counter chamber under microscope. Under an alternative condition, cells harboring plasmids containing the *ers1*, *ers2* or *Irs1* gene were incubated at 30^0C for indicated days in nitrogen free PM plates that contained 0.5% glucose. A minimum of three individual colonies from each strain was re-suspended in water and 1,000 cells/colony were microscopically examined for mating cells and ascospores. The mating and sporulation efficiency was calculated using following equation:

Mat (%) = $(2Z + 2A + 0.5S)/(H + 2Z + 2A + 0.5S)$ where Z stands for the number of Zygotes, A for the number of asci, S for the number of free spores, and H for the number of cells that failed to mate.

Measurement of cell viability. Cells were grown to $1x10^7$ cells/ml in PM medium with appropriate supplements at 30^0C. The cultures were maintained at this density and the daily intervals an aliquot was removed and plated onto indicated medium for incubation at 30^0C. The colonies formed were counted at indicated days.

Results

GluRS interacted with Moc3 in a two hybrid system

We isolated the gene named *ers2* encoding putative mitochondrial glutamyl tRNA synthetase (mGluRS) (SPAPB1A10.11c) as one of Moc3

interacting elements by yeast two hybrid system. This protein was found to be 38.5% identical to *S. cerevisiae MSE1* which encodes mitochondrial glutamyl tRNA synthetase. To further verify the consistency of interaction, we constructed pGBKT7-mGluRS which bears the *ers2* gene and Gal4 DNA binding domain (BD). mGluRS fused to the DNA BD of Gal4p was able to activate transcription of the reporter genes when present with Moc3 fused the Gal4 AD (Table 3). However mGluRS was not able to activate transcription of reporter genes when co-expressed with the Gal4 AD alone, suggesting that the interaction of mGluRS with Moc3 was specific. Similarly, we also checked whether the cytoplasmic glutamyl tRNA synthetase (SPAC17A5.15c, cGluRS), which is 35.6% similar to mGluRS and 52.6% similar to *S. cerevisiae* (*GUS1*) at amino acid level, interacts with Moc3 or not. In this regard we constructed pGBKT7-cGluRS where cGluRS was fused to Gal4 BD and checked the interaction with pGAD424-moc3 where Moc3 were fused to Gal4 AD. We found that cGluRS also interacted with Moc3 in a yeast two hybrid system (Table 3). These observations that Moc3 interacted with both mGluRS and cGluRS lead us to test their roles in sexual differentiation.

Table 3

GluRSs are essential for S. pombe growth.

We next determined whether the *S. pombe ers2* gene is essential for growth or not. A diploid *S. pombe* strain SKP17 (*ers2*$^{+}$/Δ*ers2::kanMX6*) was constructed in which one copy of *ers2* was disrupted. The diploid disruptant SKP17 was picked up from YE plate and diluted into 300µl dH_20 and plated onto PMLU medium for 3 days at 30^0C. After growing cells, colonies were treated with iodine vapor for 7-8 minutes where a few colonies were turned into brown color that sporulated by

spontaneous conversion of h^+ to h^{90}. The brown colonies were picked up and again streaked onto PMLU plates for 4 days and later tetrads were dissected through microscope and analyzed. Nine asci were dissected. Spores from the same tetrad were arrayed vertically and the plate was incubated for 4 days at 30^0C for analyzed. Among four spores only two spores formed visible colonies in YES plate (Fig. 1A) and that did not grow well in YES+G418 plates (data not shown). They were all *ers2*$^+$ spores so that they could not show resistance on the G418 plate. However, microscopic observation of dead colonies revealed that Δ*mGluRS::kanMX6* spores were incapable of forming visible colonies although capable to germinate. The Δ*ers2::kanMX6* spores divided several times before they ceased growing (Fig. 1B, C). This strongly indicates that the *ers2* gene is an essential gene for growth of *S. pombe*. This result leads us to test whether cytoplasmic glutamyl tRNA synthetase (cGluRS) is essential or not. Using the above techniques the tetrads were dissected and analyzed using *S. pombe* strain SKP19 (*ers1*$^+$/Δ*ers1::kanMX6)*. The tetrads showed a 2+:2- segregation for spore viability, and no tetrads with more than two viable spore were observed (data not shown)**.** All viable spores were sensitive to G418, indicating Δ*ers1::kanMX6* spores were not viable. Our microscopic observation indicated that the Δ *ers1::kanMX6* spores failed to germinate at all. Thus it revealed that *S. pombe* cGluRS is essential for growth as it was shown that its orthologous gene *GUS1* of *S. cerevisiae* is essential for growth.[32)] On the contrary, while *S. pombe* mGluRS was found to be essential in this study, mGluRS of *S. cerevisiae* is not essential.[33)] We will discussion on this difference in *S. pombe* and *S. cerevisiae* later. Fig.1

GluRS genes affect cell growth

We further checked the effect of *ers2* over expression on the growth of *S. pombe*. We used *S. pombe* wild type SP870 cells which over expressed the *ers2* gene under the *nmt*** promoter on the vector pREP81 in compare with SKP17-H1 (Δ*ers2::kanMX6* pREP81-mGluRS) cells under de-repressed (-Thiamine) and repressed (+Thiamine) conditions and we also used SP870 cells that over expressed the *ers2* gene under the *nmt1* promoter on the vector pREP1 that are widely used for ectopic expression and have 80-fold thiamine dependent transcriptional repression. [27] The promoter *nmt1* is turned off in the presence of thiamine.[28] Firstly, we checked the cell growth in liquid medium where we used SKP17-H1 (Δ*ers2::kanMX6* pREP81-mGluRS) cells under de-repressed (-Thiamine) and repressed (+Thiamine) conditions between SP870 over expressed *ers2* by weakest promoter and cells containing an empty vector under same conditions. Higher expression of *ers2* under de-repressed condition resulted in slow growth however cells grew faster under repressed conditions (Fig. 2A). The result indicates that cell growth severely affected by *ers2* over expression under de-repressed conditions however under repressed conditions cells multiply quickly (Fig. 2A). These result implies that a large amount of mGluRS is inhibitory for cell growth. We further checked whether cytoplasmic glutamyl tRNA synthetase (cGluRS) and other cytoplasmic tRNA synthetase like cytoplasmic leucyl tRNA synthetase (lrs1) affect on cell growth or not. In this regard, we verified the above result using *S. pombe* wild type strain SP870 harboring pREP1, pREP1-mGluRS, pREP1-cGluRS and pREP1-lrs1 plasmids. Our observation revealed that under de-repressed conditions mGluRS severely affected *S. pombe* growth whereas cGluRS and lrs1 moderately affected the cell growth (Fig. 2B). Our result clearly indicates that mGluRS has an inhibitory

effect on cell growth of *S. pombe*.

Fig.2

mGluRS gene affects on cell viability

Effect of the *ers2* gene on cell viability was also checked through counting the germinated cells. SKP17-H1 cells were cultured in PMAU medium under de-repressed and repressed conditions. Cells were counted by the cell counter at indicated times, diluted and plated on PMAU medium at 30^0C for 4 days. After 4 days germinated cells were counted. The result revealed that under de-repressed conditions cells showed low viability than repressed conditions (Fig. 3). This result indicates that the *ers2* gene might have toxic effect that inhibited the growth that leads to decreased cell viability.

Fig.3

GluRS genes induces sexual differentiation

We further checked the effect of GluRS over expression on sexual differentiation of *S. pombe* in comparison with empty vector and cells over expressing *lrs1*. We used pREP1-mGluRS, pREP1-cGluRS and pREP1-lrs1 that bears *ers2, ers1* and *lrs1* genes respectively. The cells were streaked onto PMAU-N+0.5%G (glucose) plates and mating rates were counted at indicated times. The result revealed that cells over expressed *ers2* showed a higher mating rate than cells harboring empty vector and cells over expressing *lrs1* (Fig. 4B). That result means GluRS genes have inducing ability on mating and sporulation. We also counted the mating rate in PMAU-N+0.5%G liquid medium under de-repressed and repressed conditions using SP870 cells harboring empty vector and cells over expressing *ers2*. Here we used the pREP81 vector that is expressed under the *nmt1*** promoter for *ers2* expression. The cells over expressing mGluRS showed the higher mating efficiency than cells

harboring empty vector (Fig. 4C). Moreover, we observed the mating rates using SKP17-H1 cells in the presence and absence of thiamine. The results indicated that in all cases the mating efficiency decreased drastically in the presence of thiamine. We also observed that thiamine delay and decrease the agglutination in the medium. Thiamine is metabolized to thiamine diphosphate and known to be an inhibitor of zygote formation in fission yeast.[37] This is consistent with our observation (Fig. 4C). The above observations lead us to test the effect of different amino acids on sexual differentiation on *S. pombe*.

Fig.4

Effect of amino acids on sexual differentiation

The life cycle that leads to sexual differentiation of *S. pombe* is regulated by the nutrition. The most efficient nutritional signal for induction of sexual differentiation is nitrogen starvation. Although we generally expect that amino acids increase the nutritional availability, we checked whether any additional amino acid can induce sexual differentiation or not. *S. pombe* wild type strain SP870 were grown on PM medium (contain 0.5% NH_4Cl with appropriate supplements) with 100mg/l and 250mg/l amino acids and incubated at 30^0C for 2 days (Fig. 5A, B). Our results indicated that glutamic acid, tryptophan and threonine efficiently induced sexual differentiation including methionine as that was previously shown to have an inducible ability on mating.[35] We noticed glutamic acid with 250mg/l at the 24hr point is the highest inducing ability on mating (Fig. 5B). On the contrary leucine showed an inhibitory effect on mating in nitrogen rich PM medium (Fig. 5A). But this was not the case under nitrogen limiting conditions (data not shown). We verified these results using wild type *S. pombe* strain L968 (FY7520) to check the strain dependency. This strain is the very

wild type h^{90} strain which does not retain any auxotrophic markers. In PM medium containing 0.01% NH_4Cl, mating and sporulation of L968 were efficiently induced by tryptophan, methionine, threonine and glutamic acid, when individual amino acid was added in the medium (Fig. 5C). Thus, those four amino acids consistently induced mating of two tested strains under our experimental conditions.

Fig.5

Discussion

In this study, we isolated a novel gene which encodes mGluRS as a Moc3 interacting element from *S. pombe* cDNA library by yeast two hybrid system and found cytoplasmic GluRS also interacted with Moc3. Moc3 plays a positive role to induce sexual differentiation but its exact role is not clear.[20] Although the biological significance of the interaction of Moc3 with mGluRS or cGluRS was not clear, we though it would be interesting to explore the role of mGluRS or cGluRS on sexual differentiation. While Moc3 was shown to localize in the nucleus,[20] cGluRS localizes in the cytosol and the nucleus [36] and mGluRS localizes in the mitochondria.[36] From those localization observation, the interaction of Moc3 with cGluRS is more likely than with mGluRS. In fact some aminoacyl tRNA synthetases are shown to localize in the nucleus and involves in tRNA maturation in other species. [37-38]

Our result showed that disruption of the *ers2* gene was lethal and terminal phenotype of the disruptant showed that cells divided several times before they ceased growing (Fig. 1B, C). The cytoplasmic glutamyl tRNA synthetase (cGluRS) gene is also essential for *S. pombe* and Δ*ers1* spores were incapable to germinate. These results are the first examples in fission yeast that showed aminoacyl tRNA synthetase is essential for growth among 40 putative aminoacyl tRNA synthetases on

the genome [http://www.genedb.org/genedb/pombe]. Essentiality of *ers2* and *ers1* genes is reasonable because mGluRS and cGluRS contribute in the mitochondrial and cytoplasmic protein synthesis. Due to disruption of mGluRS and cGluRS genes, the translational activity will be completely lost that leads to hamper the protein biosynthesis. Aminoacyl-tRNA synthetases are required for faithful translation of the genetic code, as to catalyze charging of tRNAs with their cognate amino acids.[37-38] It is notable that the mGluRS ortholog in *S. cerevisiae MSE1* is not essential for growth[32] while the *ers1* ortholog *GUS1* is essential for budding yeast.[33] This difference of mGluRS requirement for fission yeast and budding yeast reflect the dependency of mitochondria in both yeasts. *S. pombe* is considered a petite negative and *S. cerevisiae* a petite positive yeast. “Petite negative” has been defined as the inability (or near-inability) to lose mitochondrial DNA. Because deletion of *MSE1* in *S. cerevisiae* leads to mitochondrial DNA instability, [32] deletion of *ers1* in *S. pombe* is expected to result in the same consequence. One reason why *S. pombe* is petite negative may be related to its primarily aerobic metabolism related to mitochondrial functions. Especially, respiration is very important for the growth of fission yeast.[39,40]

We also noticed, the higher expression of the *ers2* gene resulted in slow growth (Fig. 2A, B) and decreased the cell viability (Fig. 3). Moreover, Ers1 and lrs1 has moderate effect on growth (Fig. 2B). This phenomenon is interesting because GluRS genes are essential for growth but when their expressions were increased they hampered the growth and mGluRS decrease the viability of fission yeast. It is occasionally observed that over production of mitochondrial protein in fission yeast is inhibitory for growth.[40]

GluRS (mGluRS and cGluRS) induces sexual differentiation of *S.*

pombe when these genes were over expressed (Fig. 4A, B, C) whereas lrs1 over expression did not show significant effect on sexual differentiation (Fig. 4B). This difference is quite interesting if combined with the current results that additional glutamic acid induced sexual differentiation meanwhile leucine rather lowed the mating rate (Fig. 5). Those results predict that the involvement of some specific amino acids on protein synthesis may relate to sexual differentiation. These results also suggest that some amino acids are not only the source of nitrogen but also the inducers of sexual differentiation in fission yeast. These amino acids might interfere some pathways that caused the nitrogen starvations in cells. It is often observed that mutations in some genes involved in amino acids metabolism affect the mating efficiency of fission yeast (our observation). Methionine is an important metabolite in fission yeast cells. The addition of methionine was shown to induce sexual differentiation *via* an *ste11*-dependent signaling pathway in fission yeast.[35] We were able to repeat this methionine effect (Fig. 5), and we also showed that other amino acids such as glutamic acid, trptophan and threonine have positive effects on sexual differentiation. It is yet not clear why those selected amino acids have the positive effects of inducing sexual differentiation, but there are some relevant examples in other species. Among natural amino acids, L-glutamic acid is considered to play an important role as a sex inducing pheromone and work as a mediator of sexual differentiation in the green alga *Volvox capensis*.[41] Studies in *S. cerevisiae* suggested that glutamic acid may play an important role in the transition between the mitotic (vegetative) and meiotic (sporulative) stages of the life cycle.[42-43] Glutamic acid is the major metabolite produced shortly after yeast cells have been triggered to sporulate in acetate, and sporulation de-repressed mutants

produced abnormally high level of glutamic acid in *S. cerevisiae*.[44] Those observations in other species may indicate some common role of glutamic acid in sexual differentiation.

In conclusions, the GluRS genes are essential for *S. pombe* growth and a large amount of these genes is inhibitory for cell growth at different degrees. The GluRS gene induces sexual differentiation of *S. pombe*. Glutamic acid has inducing ability on sexual differentiation. These results predict a new regulatory mechanism through glutamic acid and its tRNA synthetase on sexual differentiation. More analysis will be required to know the mechanism.

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Figure legends

Fig. 1. Disruption of the *GluRS* gene.

A, Tetrad analysis of *ers2* disruptants. SKP17 (*ers2*$^{+}$/Δ *ers2::kanMX6*) clones were subjected for tetrad analysis. Tetrads were dissected on YES medium, and spores were grown at 30^{0}C for 4 days. Nine tetrads were dissected and four spores from each tetrad were aligned vertically. B and C, Terminal phenotype of Δ*ers2* spores at 30^{0}C for 4 days.

Fig. 2. *GluRS* gene affects cell growth.

A, Growth assay of the haploid *ers2* disruptant harboring pREP81-mGluRS (SKP17-H1) in comparison with the strain that over expressed mGluRS on pREP81-mGluRS and the one harboring empty vector under de-repressed and repressed conditions. Cell number was counted using the Cell counter (Sysmex) at indicated times. B, Growth assay of cells over expressing mGluRS, cGluRS, or lrs1 in comparison with cells harboring empty vector under de-repressedand repressed conditions. The *ers1, ers2* and *lrs1* genes were over expressed on pREP1-mGluRS, pREP1-cGluRS, pREP1-lrs1 that bears respective gene and thiamine repressible strong *nmt1* promoter.

Fig. 3. *mGluRS* gene affects on cell viability.

SKP17-H1 cells were cultured in PMAU medium under de-repressed (-Thiamine) and repressed (+Thiamine) conditions until the indicated days. Cell number was counted by cell counter (Sysmex) at indicated times, then immediately diluted and plated onto PMAU medium that were incubated at 30^{0}C for 4 days. The germinated cells

were counted by counting the visible colonies and converted to viability percentage for indicated days.

Fig. 4. *GluRS* gene affects sexual differentiation of *S. pombe*.

A, Microscopic observation of SP870 cells harboring pREP1, pREP1-mGluRS, or pREP1-cGluRS after 24 hours cultured on PMAU-N+0.5% plate. Wild type SP870 cells were transformed with pREP1, pREP1-mGluRS or pREP1-cGluRS cultured onto PMAU plates at 30^0C for 3 days. Single colony was picked up and streaked onto PMAU-N+0.5% glucose plate at 30^0C. A single colony was picked up and dissolved in water at 24 hours incubation. After vortexing and sonication, pictures were taken. B, Mating rates of cells over expressing *ers2*, *ers1* and *lrs1* in compare with empty vector harboring cells at indicated times. Here, *ers2*, *ers1* and *lrs1* were over expressed on pREP1-mGluRS, pREP1-cGluRS, pREP1-lrs1 that bears respective gene and thiamine repressible strong promoter. Three individual colonies were picked up at indicated times and dissolved in water. Mating efficiency of 1,000 cells was counted. The mating efficiency are plotted in the bar diagram as a percent of total cells. C, Mating efficiency of the haploid *ers2* disruptant harboring pREP81-mGluRS (SKP17-H1) and cells over expressing *ers2* in comparison with vector. Here, the *ers2* gene was over expressed on pREP81-mGluRS by thiamine repressible weakest promoter (*nmt1***). Cells were grown in PMAU liquid medium until mid log phase. Then the cells were harvested and washed three times with nitrogen free medium and cultured into nitrogen free medium (PMAU-N+0.5% glucose) at 30^0C for indicated times. Cells were observed under microscope and mating efficiency was counted among 1,000 cells.

Fig. 5. Effect of amino acid on sexual differentiation of fission yeast.

A, B, *S. pombe* wild type strain SP870 were pre-cultured using PMALU liquid medium at 30^0C. From the first pre-culture cells were transferred to second pre-culture. When cells reached at mid-log phase then cells were again shifted to PMALU medium and incubated to allow mating. Amino acids were added in PMALU medium at 100mg/l (A) and 250mg/l (B) and the mating cells were counted after 24 and 48 hours intervals. C, D, *S. pombe* wild type strain FY7520 were pre-cultured using PM (0.5% NH_4Cl) liquid medium at 30^0C. From the first pre-culture cells were transferred to second pre-culture. When cells reached at mid-log phase then cells were again shifted to PM (0.01% NH_4Cl) medium and incubated to allow mating. Various amino acids were added at 250mg/l (C) in PM (0.01% NH_4Cl) medium and counted the mating cells after 24 and 48 hours intervals.

Table 1. List of *S. cerevisiae* and *S. pombe* strains used in this study

Strain	Genotype	Source
AH109	*MATa, trp1-901, leu2-3, ura3-52, his3-200, gal4Δ gal80Δ, LYS2: : GAL1* $_{UAS}$- GAL1 $_{TATA}$ -HIS3, GAL2 $_{UAS}$ - GAL2 $_{TATA}$-ADE2, URA: : MEL1 $_{UAS}$ *-MEL1* $_{TATA}$*-LacZ*	Lab. stock
SP870	h^{90}*ade6.210 leu1.32 ura4-D18*	45)
SP826	h^{+}*leuI.32 ade6.210 ura-D18/*h^{+}*leuI.32 ade6.216 ura4-D18*	45)
FY7520(L968)	h^{90}	NBRP/YGRC
SKP17	h^{+}*leuI.32ade6.210ura-D18/*h^{+}*leuI.32ade6.216 ura4-D18 ers2::kanMX6*	This study
SKP17-H1	h^{90}*leu1.32ade6.210ura4-D18 ers2::kanMX6 (pREP81-mGluRS)*	This study
SKP19	h^{+}*leuI.32ade6.210ura4-D18/*h^{+}*leuI.32ade6.216 ura4-D18 ers1::kanMX6*	This study

Table 2. List of oligonucleotide primers used in this study

pGBKT7/pGAD424	
moc3-F-SmaI	5'-CCTCCCGGGTATGAACCCGTATGTTTCTTATC-3'
moc3-R-Sal1	5'-CCAGTCGACTGACTGTCGTACCGTAATTCG-3'
pGBKT7	
mGluRS-F-EcoRI	5'-TTTGAATTCATGCTCTCTTATACCTC-3'
mGluRS-R-SalI	5'-GGTTAGGTCGACTTATACGATACAACTTC-3'
cGluRS-F-BamHI	5'-GTTGGATCCAAATGTCAGTTAGTGTTGC-3'
cGluRS-R-PstI	5'-TGCCTGCAGCTAATTTTTAACGCCGTAAC-3'
pREP1/ pREP81	
mGluRS-F-SalI	5'-ATTGTCGACGATGCTCTCTTATACCTC-3'
mGluRS-R-BamHI	5'-GGTTAGGGATCCTTATACGATACAACTTC-3'
cGluRS –F-BamHI	5'-GTTTGGATCCAATGTCAGTTAGTGTTGC-3'
cGluRS -R-SmaI	5'-TGCCCCGGGCTAATTTTTAACGCCGTAAC-3'
Lrs1-F-NdeI	5'-TCCCATATGGCTACGACTGAACC-3'
Lrs1-R- SalI	5'-CAAGTCGACTTAAGCAGAGACATTTTC-3'
mGluRS disruption primers	
mGluRS (A)	5-ATGGCTTTCCCTTCGTAAAC-3'
mGluRS (B)	5'-GGGGATCCGTCGACCTGCAGCGTACGACCAGAATTGGATATAAGGTTGC-3'
mGluRS (Y)	5'-GTTTAAACGAGCTCGAATTCATCGATCAATGCGTTTTACGGAAAGG-3'
mGluRS (Z)	5'-CACTACACCATCAATGGTTG-3'
cGluRS disruption primers	
cGluRS (A)	5-AGGTGCTCTTTTTCTACTCTC-3'
cGluRS (B)	5'-GGGGATCCGTCGACCTGCAGCGTACGAACTTGGTTCGTGGTAAG-3'
cGluRS (Y)	5'-GTTTAAACGAGCTCGAATTCATCGATTGTATCTGAGACTAAACTCCG -3'
cGluRS (Z)	5'-CTTTTCCATCACCTCCATCC-3'

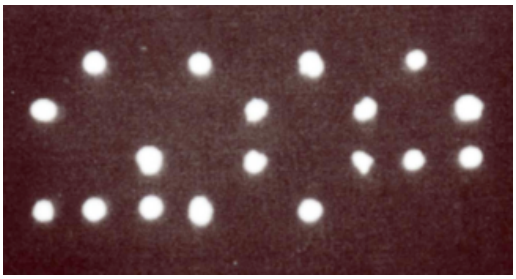
Restriction enzyme sites are underlined

Table 3. Interaction of GluRS with Moc3 by yeast two hybrid system

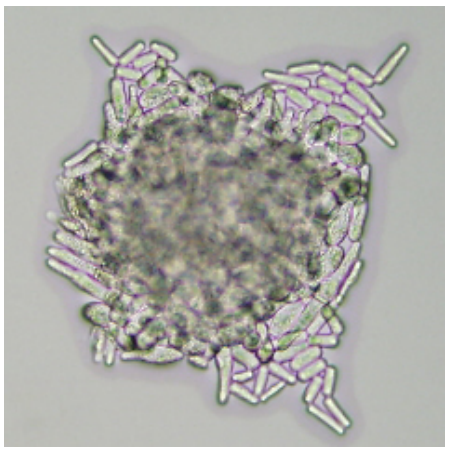
DNA-binding (pGBKT7)	DNA-activating (pGAD424)	X-gal
Empty	Empty	-
mGluRS	Empty	-
mGluRS	Moc3	++
cGluRS	Empty	-
cGluRS	Moc3	+
P53	T-Antigen	+++

Positive signal indicated by '+' and negative signal as '-' symbol

A



B



C

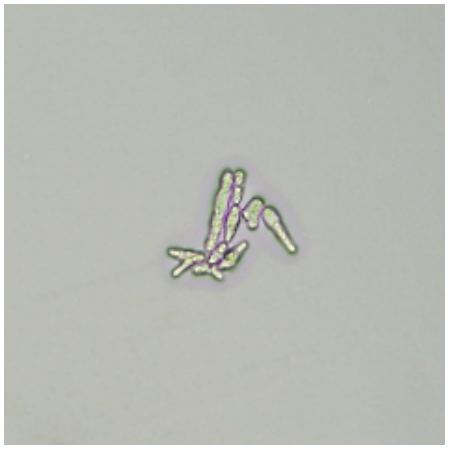
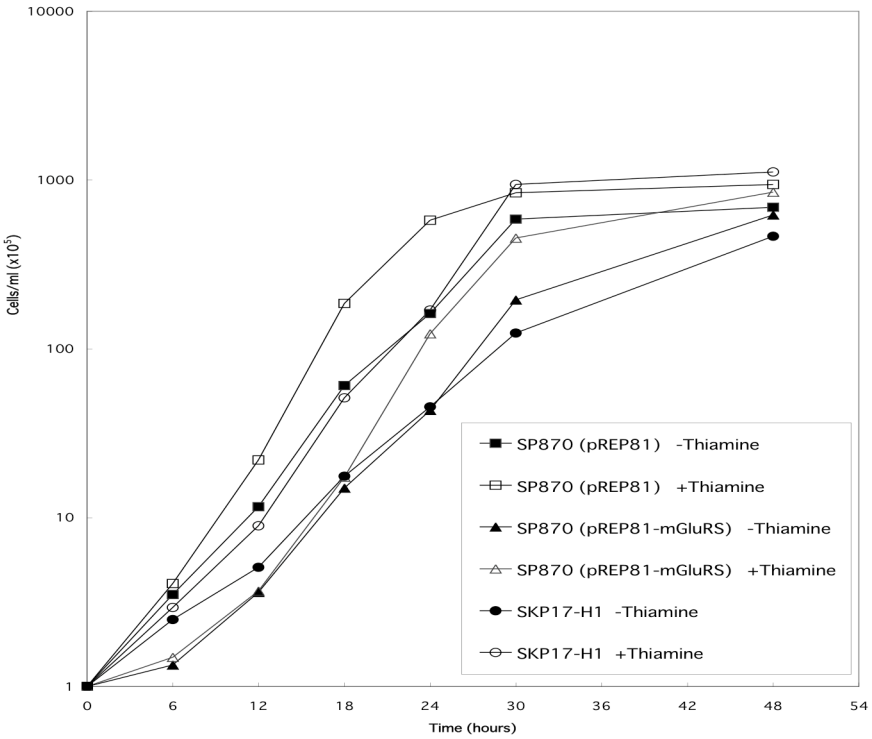


Fig. 1.

A


10000
1000
100
10
1
Cells/ml (x10^5)
0
6
12
18
24
30
36
42
48
54
Time (hours)
SP870 (pREP81) -Thiamine
SP870 (pREP81) +Thiamine
SP870 (pREP81-mGluRS) -Thiamine
SP870 (pREP81-mGluRS) +Thiamine
SKP17-H1 -Thiamine
SKP17-H1 +Thiamine

B

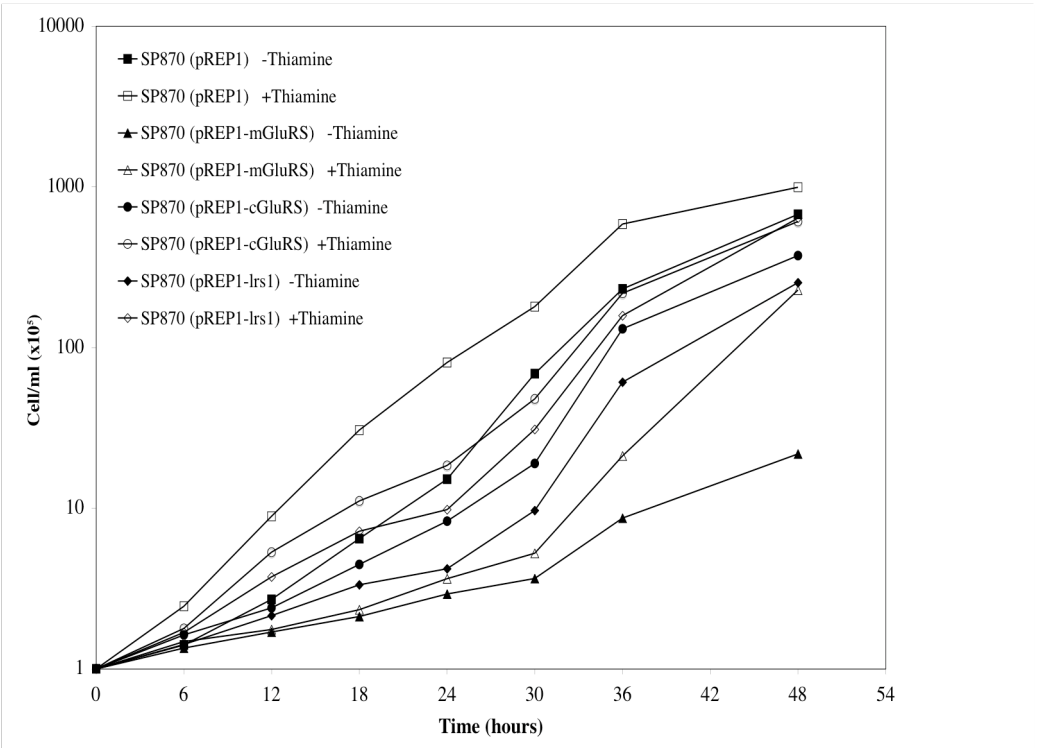

10000
1000
100
10
1
Cell/ml (x10^5)
0
6
12
18
24
30
36
42
48
54
Time (hours)
SP870 (pREP1) -Thiamine
SP870 (pREP1) +Thiamine
SP870 (pREP1-mGluRS) -Thiamine
SP870 (pREP1-mGluRS) +Thiamine
SP870 (pREP1-cGluRS) -Thiamine
SP870 (pREP1-cGluRS) +Thiamine
SP870 (pREP1-lrs1) -Thiamine
SP870 (pREP1-lrs1) +Thiamine

Fig. 2.

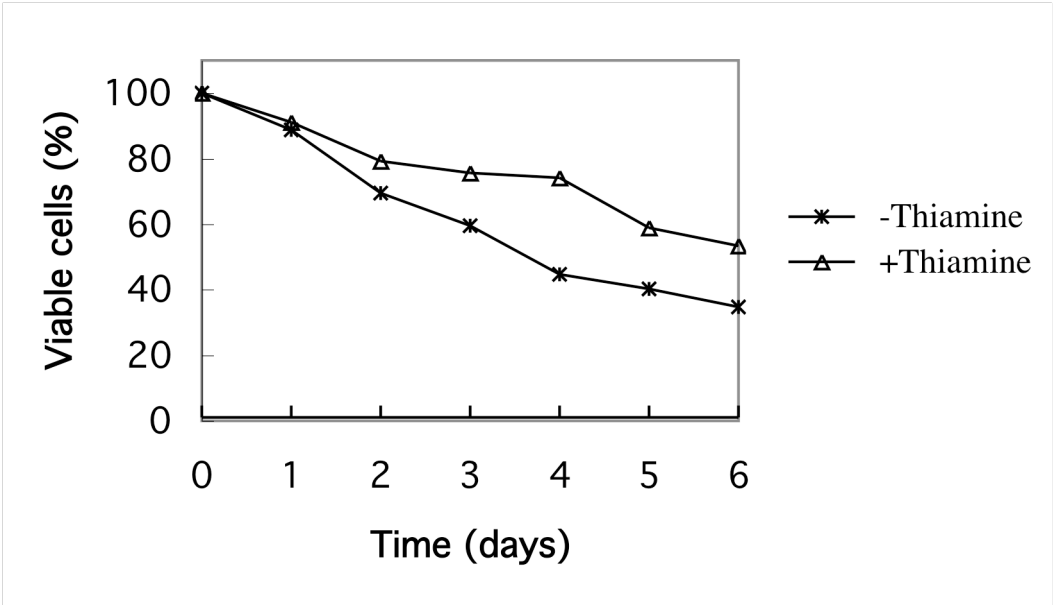
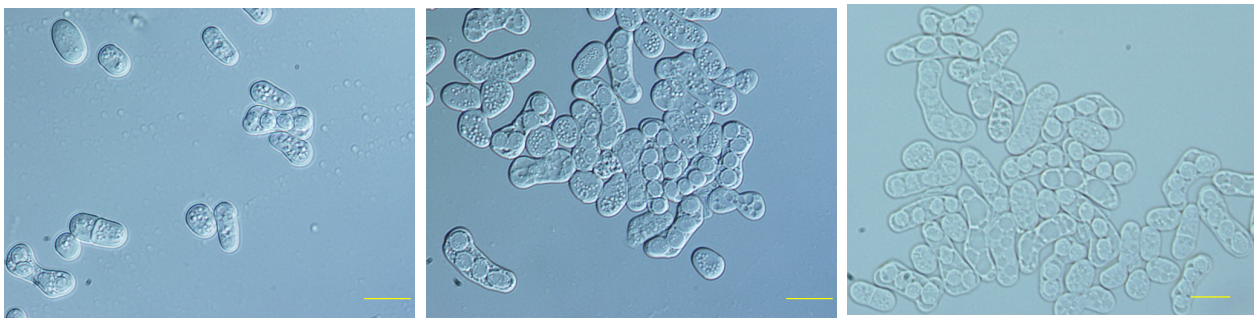

Viable cells (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (days)
-Thiamine
+Thiamine

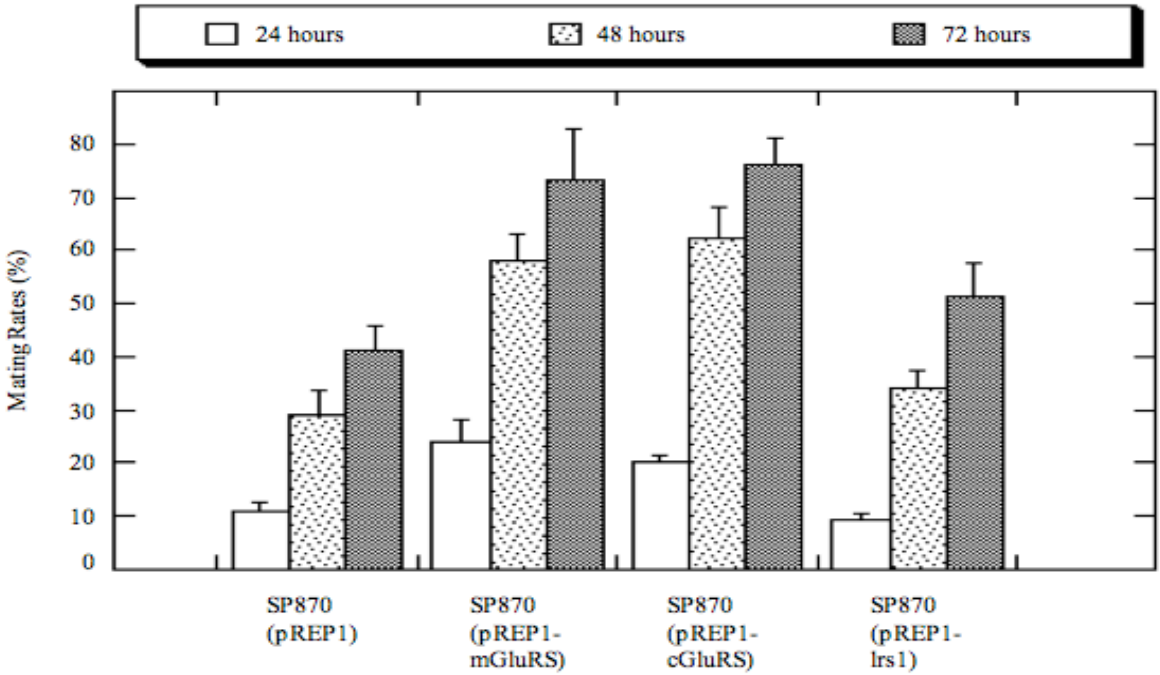
Fig. 3.

A



SP870 (pREP1) SP870 (pREP1-mGluRS) SP870 (pREP1-cGluRS)

B



C

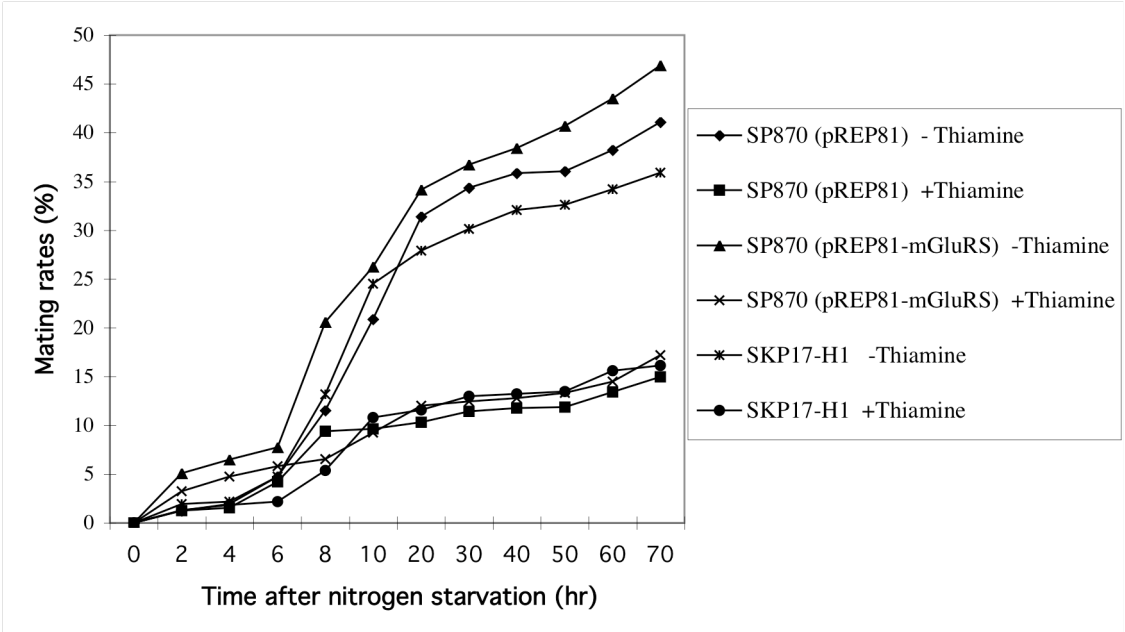


Fig. 4.

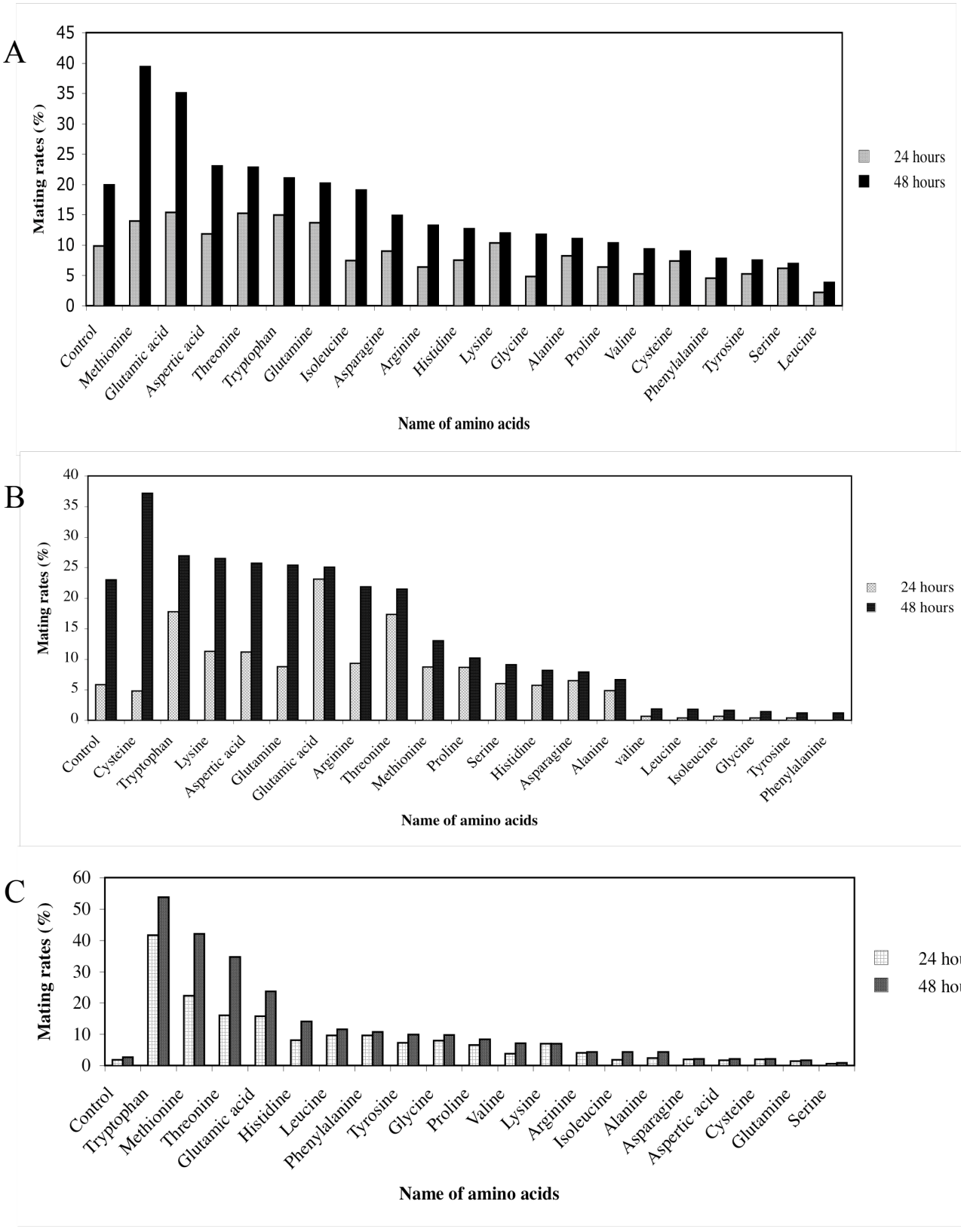

A
Mating rates (%)
45
40
35
30
25
20
15
10
5
0
24 hours
48 hours
Control
Methionine
Glutamic acid
Aspertic acid
Threonine
Tryptophan
Glutamine
Isoleucine
Asparagine
Arginine
Histidine
Lysine
Glycine
Alanine
Proline
Valine
Cysteine
Phenylalanine
Tyrosine
Serine
Leucine
Name of amino acids
B
Mating rates (%)
40
35
30
25
20
15
10
5
0
24 hours
48 hours
Control
Cysteine
Tryptophan
Lysine
Aspertic acid
Glutamine
Glutamic acid
Arginine
Threonine
Methionine
Proline
Serine
Histidine
Asparagine
Alanine
valine
Leucine
Isoleucine
Glycine
Tyrosine
Phenylalanine
Name of amino acids
C
Mating rates (%)
60
50
40
30
20
10
0
24 hours
48 hours
Control
Tryptophan
Methionine
Threonine
Glutamic acid
Histidine
Leucine
Phenylalanine
Tyrosine
Glycine
Proline
Valine
Lysine
Arginine
Isoleucine
Alanine
Asparagine
Aspertic acid
Cysteine
Glutamine
Serine
Name of amino acids

Fig. 5.