Bioengineered 3:5, 293–297; September/October 2012; © 2012 Landes Bioscience

Extremotolerant fungi as genetic resources for biotechnology

Cene Gostinčar[1,2] and Martina Turk[1,*]

[1]Department of Biology; Biotechnical Faculty; University of Ljubljana; Ljubljana, Slovenia; [2]Centre of Excellence for Integrated Approaches in Chemistry and Biology of Proteins (CIPKeBiP); Ljubljana, Slovenia

Increased stress tolerance of economically important plants and microorganisms can improve yields in agriculture and industrial microbiology. The pool of resources used for the genetic modification of crops and industrial fungal strains in the past has been relatively limited, and has frequently included only stress-sensitive organisms. However, certain groups of fungi have evolved specialized mechanisms that enable them to thrive under even the most extreme of environmental conditions. These species can be considered as promising sources of biotechnologically interesting genes. Together with a powerful and convenient high-throughput functional screening method, extremotolerant fungi represent a new opportunity for the identification of stress-tolerance-conferring genes. The approaches described here should provide important contributions to the enhancing of the properties of economically important organisms in the future.

Keywords: functional screening, gain-of-function method, stress tolerance genes, stress-tolerant fungi, halotolerance, extremotolerant organism, genetically modified organism, salinization

Submitted: 04/26/12

Revised: 05/10/12

Accepted: 05/10/12

http://dx.doi.org/10.4161/bioe.20713

*Correspondence to: Martina Turk;
Email: martina.turk@bf.uni-lj.si

Addendum to: Gostincar C, Gunde-Cimerman N, Turk M. Genetic resources of extremotolerant fungi: A method for identification of genes conferring stress tolerance. Bioresour Technol 2012; 111:360–7; PMID:22386631; http://dx.doi.org/10.1016/j.biortech.2012.02.039.

Genetic modifications are one of the most promising methods for the improvement of economically important organisms. Recently the required methodology has advanced substantially and in the three decades since insulin produced through recombinant technology arrived on the market, genetically modified organisms have truly become our reality. The sources of the genes to be introduced, however, have remained relatively limited, and this might present increasing problems when there is the need to improve complex traits, such as tolerance to different types of abiotic stress.

Crops with increased tolerance to drought and high salinity can substantially increase the productivity in agriculture and alleviate the problems of limited water supplies. Agriculture is one of the main factors behind the increasing global scarcity of fresh water.[1] This water scarcity, which is typically accompanied by increased salinity of the soil (which is also due to poor irrigation practices), is the most common cause of food shortages in developing countries.[2] Global climate changes are further increasing the frequency of droughts and the need for irrigation, and the subsequent salinisation is reducing the existing areas under irrigation by 1–2% per year.[3] At the same time, agricultural yields, lack of water, and other types of abiotic stress also affect industrial microbiology. For example, during ethanol fermentation, the cells of *Saccharomyces cerevisiae* are exposed to osmotic, oxidative and thermal stress, and to high ethanol concentrations, and/or starvation.[4] This is increasingly important in the production of bioethanol, a technology that can reduce our dependence on fossil fuels. The ethanol market yield is expected to reach 100 billion liters in 2015.[5] New fermentation techniques, such as very high gravity fermentation or fully aerated batch-fed fermentation, are more effective than traditional anaerobic or microaerobic fermentation, but at the same time, these expose the cells to greater levels of stress, like low water activity and high concentrations of ethanol.[5,6] Yeast strains with higher stress tolerance are thus essential for improved ethanol productivity.[7]

With these facts in mind, it is not surprising that increased tolerance to high salt concentrations or lack of water in plants and industrial microorganisms through genetic engineering is highly desirable, especially in the light of the

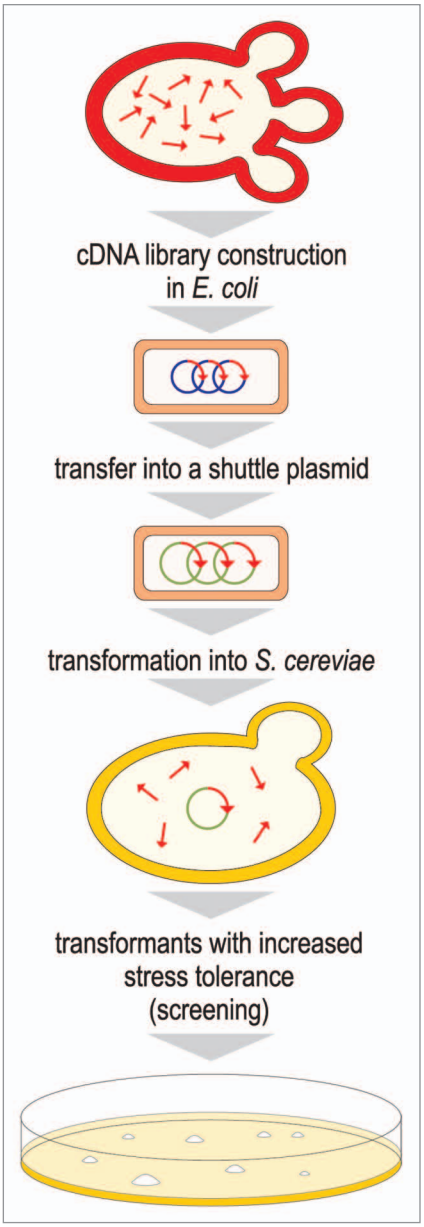

Figure 1. Schematic representation of functionctional screening of a cDNA library from a stress-tolerant fungus in *Saccharomyces cerevisiae*.

limited success of traditional breeding approaches.[8] Several genes have been used for this purpose so far, but many of these have originated from relatively stress-sensitive organisms. For example, drought tolerance genes from fungi for improvement of crops have been derived mainly from *Saccharomyces cerevisiae*. The need for novel sources of stress tolerance genes has already been recognized.[9] We believe that naturally stress-tolerant organisms from extreme environments can be considered as the gene donors of the future. Certain groups of fungi are among the most promising candidates. However, while fungi have long been accepted as a good source of extracellular-enzyme-encoding genes,[10] in other fields, their resources have been less well exploited. Although certain fungal groups are extremely well adapted to hypersaline environments,[11] and also to a variety of other stress conditions (reviewed by Gostincar et al.[12]), there are no published studies describing functional screening of halotolerant fungi for genes that can be used to confer halotolerance—with one exception. Increased halotolerance was described in several clones of *S. cerevisiae* transformed with a genomic library prepared from the halotolerant yeast *Debaryomyces hansenii*.[13] However, the genes responsible for the increased tolerance were not reported.

To facilitate the identification of such genes of potential biotechnological interest, a new high-throughput screening approach has been proposed.[14] This involves the construction of a plasmid cDNA library using a commercial kit, the transfer of the library to an *E. coli*—*S. cerevisiae* shuttle plasmid vector by homologous recombination, the transformation into yeast, and the screening of this expression yeast library for gain-of-function transformants (**Fig. 1**). This approach has several advantages: (1) Naturally extremely stress-tolerant organisms are used as gene donors. These organisms have evolved specialized mechanisms to combat the harsh conditions in their habitats, and are a more suitable source of stress tolerance adaptations than the previously used stress-sensitive organisms. (2) Fungi are more promising gene donors for the improving of stress tolerance of plants and industrially important fungi compared with structurally different and phylogenetically distant prokaryotes. (3) *S. cerevisiae* is a more appropriate screening system than prokaryotes, while at the same time it is still easily manipulated in the laboratory. (4) The procedure described uses commercially available kits and reagents, and it is therefore convenient and easily reproducible.

The possible problems that may be encountered with the suggested method include the known shortcomings of heterologous expression. The expression of the cloned genes may be inadequate due to differences in codon usage between the donor and the host or other factors. On the other hand, overexpressed proteins may disturb the metabolism of the cell or be toxic in other ways. It is therefore reasonable to expect that the method will not identify all of the genes that are theoretically beneficial for a given purpose. Additionally, the possibility that the increased stress tolerance is caused by the genomic mutations of the host organism, has to be taken into account. The causative link between the heterologous gene and the changed phenotype should always be confirmed with additional experiments.

The efficiency of the method has been demonstrated on the example of the stress-tolerant basidiomycetous yeast *Rhodotorula mucilaginosa*. Functional screening of the expression library with genes from this species has yielded more than 100 *S. cerevisiae* transformants that have gained the ability to grow at previously inhibitory concentrations of various osmolites. The cloned genes have been sequenced in 92 of these transformants (**Fig. 2**), and a detailed analysis of a subset of 12 transformants has confirmed the causative role of the *R. mucilaginosa* genes in increased stress tolerance. Several genes have been obtained from more than one transformant. The most redundant were those that encode two different phosphomutases, and these were shown to increase tolerance to increased concentrations of both NaCl and LiCl. Homologs of both of these enzymes have been well studied in other organisms. They catalyze important reactions in carbohydrate metabolism, and they have been evolutionarily conserved. As expected, in the reconstruction of the gene phylogenies, proteins from *R. mucilaginosa* cluster with homologs from other basidiomycetous fungi (**Fig. 3**).

Phophoglucomutase is encoded by the gene *RmPGM2*, and it catalyzes the interconversion of glucose-6-phosphate and glucose-1-phosphate. Thus, it is directly or indirectly involved in glycolysis, the pentose phosphate pathway, the metabolism of galactose and trehalose, and the synthesis of cell wall β-glucans and *N*-linked glycoproteins.[15-17] In *S.*

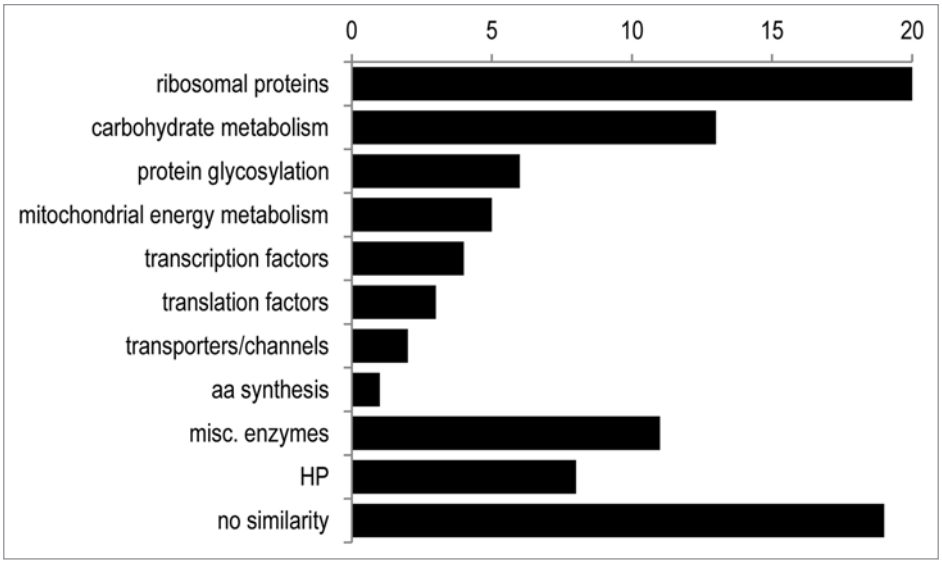

Figure 2. Functional categories of 92 genes from *Rhodotorula mucilaginosa* that increase the tolerance to elevated concentrations of osmolytes (NaCl, LiCl, glycerol and sorbitol) of the recipient *Saccharomyces cerevisiae* strain W303a. Number of proteins is shown on the X axis. HP-proteins, showing similarity only to hypothetical proteins.

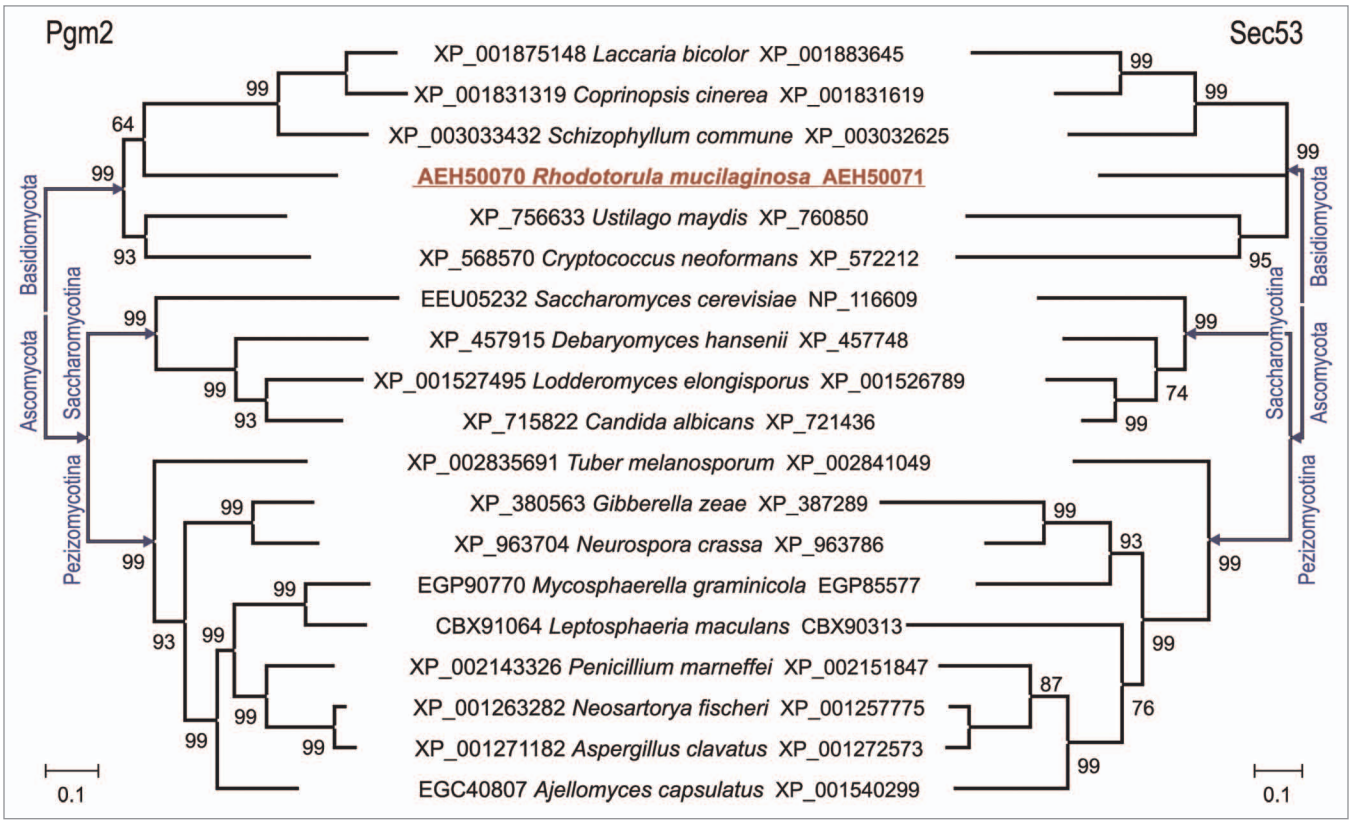

Figure 3. Gene trees of the phosphoglucomutase (Pgm2, left) and phosphomannomutase (Sec53, right) enzymes. The complete protein sequences were used for the reconstruction of the phylogeny by aligning them with Probcons[22] and by building the tree with the Phyml software.[23] The approximate likelihood ratio test was used to calculate the chi-square-based branch supports. The GenBank accession numbers are given to the left (Pgm2) and right (Sec53) of the species names.

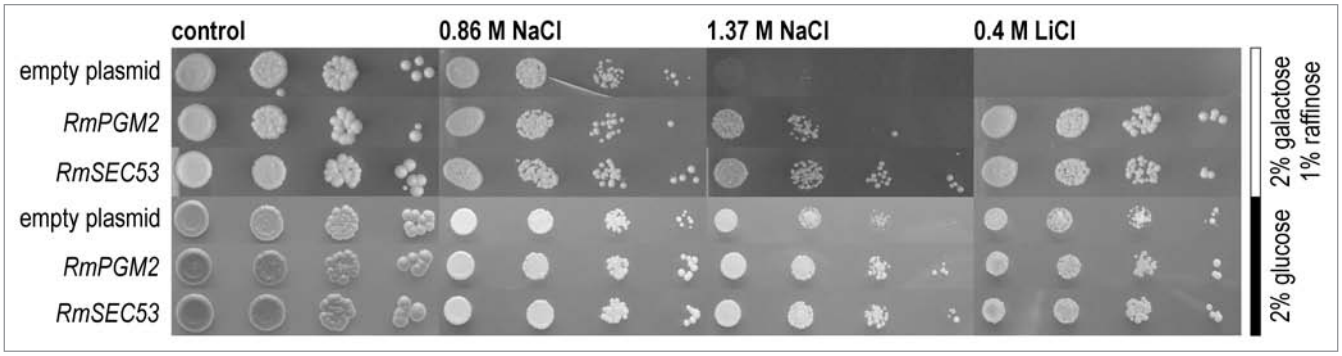

Figure 4. Increased salt tolerance of *Saccharomyces cerevisiae* W303a expressing the phosphoglucomutase (*RmPGM2*) and phosphomannomutase (*RmSEC53*) genes from *Rhodotorula mucilaginosa*. Expression was driven by the *GAL1* (on galactose/raffinose) or TPI (on glucose) promoters. YNB + CSM-Ura and YNB + CSM-Leu media with added NaCl or LiCl were used.

cerevisiae, phophoglucomutase is inhibited by lithium[18] and its expression increases in response to lithium and sodium stress[18] and heat shock, and during adaptation to cold.[19] The other of the two phosphomutases is a phosphomannomutase that is encoded by *RmSEC53*. Its homolog in *S. cerevisiae* catalyzes the conversion of mannose-6-phosphate to mannose-1-phosphate, and it is crucial for *N*-linked glycosylation of proteins and associated protein sorting and secretion.[20,21] The enzyme also has a phosphoglucomutase activity (interconversion of glucose-1-phosphate and glucose-6-phosphate[17]) and in the case of RmSec53, this activity is less sensitive to lithium than the activity of RmPgm2.[14] While the two *R. mucilaginosa* phosphomutases can increase lithium tolerance of *S. cerevisiae* in two different ways, there is also the possibility that they can both act by rescuing the inhibited phosphoglucomutase activity, an extremely important activity for the carbohydrate metabolism of the cell.

As phosphoglucomutase activity is induced during growth on galactose, and as this sugar was used in our experiments as the carbon source to induce the expression of the cloned genes, the identification of both of these phosphomutases might be an artifact caused by the screening conditions. However, this was not the case. When the genes were transferred into the pYX142 plasmid with a constitutive TPI promoter and grown on glucose, the increased halotolerance effect was still present, although less pronounced (**Fig. 4**).

The main advantages of functional gene screening like that used here are the high-throughput applicative approach that does not depend on detailed hypotheses about the biotechnological potential of specific genes, the relative independence from previous knowledge about the donor organism, and the great flexibility of the method. The method described can easily be modified for other purposes than the one described above. By using a vector with a dominant selectable marker or a constitutive promoter, the screening can be performed with industrial strains of *S. cerevisiae* or on carbon sources other than galactose. Changed screening conditions can also be used to screen for genes that confer tolerance to types of stress other than high salt or low water activity, such as extreme pH, temperatures and oxygen stress, among others. At the same time, cDNA libraries can be prepared from pure cultures as well as metatranscriptomes of stress-tolerant communities; for example, from coastal hypersaline water ponds, in the case of the search for genes that can confer halotolerance.

Extremely stress-tolerant fungi should be investigated for their biotechnological potential to improve the stress tolerance of economically important fungi and plants. This method of functional screening is a valuable tool for tapping into the significant, but currently largely neglected, genetic resources of these intriguing organisms. It is hoped that the approach described will contribute to the much needed improvement of organisms, the performance of which can influence agricultural and industrial practices on a global scale.

Acknowledgments

The scientific work was partly financed via the project "Centre of excellence for integrated approaches in chemistry and biology of proteins" number OP13.1.1.2.02.0005, financed by the European regional development fund (85% share of financing) and by the Slovenian Ministry of Higher Education, Science and Technology (15% share of financing). The authors also acknowledge the financial support from the state budget through the Slovenian Research Agency (Infrastructural Centre Mycosmo and grant no. J4-2022).

References

1. Bruinsma J. The resource outlook to 2050: By how much do land, water and crop yields need to increase by 2050? FAO Expert meeting on how to feed the World in 2050. Rome 2009.
2. Cominelli E, Tonelli C. Transgenic crops coping with water scarcity. N Biotechnol 2010; 27:473-7; PMID:20723623; http://dx.doi.org/10.1016/j.nbt.2010.08.005.
3. FAO. Crops and Drops. World Food Day. Rome 2002.
4. Ding JM, Huang XW, Zhang LM, Zhao N, Yang DM, Zhang KQ. Tolerance and stress response to ethanol in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 2009; 85:253-63; PMID:19756577; http://dx.doi.org/10.1007/s00253-009-2223-1.
5. Mussatto SI, Dragone G, Guimarães PMR, Silva JPA, Carneiro LM, Roberto IC, et al. Technological trends, global market and challenges of bio-ethanol production. Biotechnol Adv 2010; 28:817-30; PMID:20630488; http://dx.doi.org/10.1016/j.biotechadv.2010.07.001.

6. Cot M, Loret MO, François J, Benbadis L. Physiological behaviour of *Saccharomyces cerevisiae* in aerated fed-batch fermentation for high level production of bioethanol. FEMS Yeast Res 2007; 7:22-32; PMID:17005001; http://dx.doi.org/10.1111/j.1567-364.2006.00152.x.
7. Tao X, Zheng D, Liu T, Wang P, Zhao W, Zhu M, et al. A novel strategy to construct yeast *Saccharomyces cerevisiae* strains for very high gravity fermentation. PLoS One 2012; 7:31235; PMID:22363590; http://dx.doi.org/10.1371/journal.pone.0031235.
8. Bhatnagar-Mathur P, Vadez V, Sharma KK. Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. Plant Cell Rep 2008; 27:411-24; PMID:18026957; http://dx.doi.org/10.1007/s00299-007-0474-9.
9. Somvanshi VS. Patenting drought tolerance in organisms. Recent Pat DNA Gene Seq 2009; 3:16-25; PMID:19149734; http://dx.doi.org/10.2174/187221509787236228.
10. Dalbøge H. Expression cloning of fungal enzyme genes; a novel approach for efficient isolation of enzyme genes of industrial relevance. FEMS Microbiol Rev 1997; 21:29-42; PMID:9299700; http://dx.doi.org/10.1016/S0168-6445(97)00005-3.
11. Gunde-Cimerman N, Zalar P, de Hoog S, Plemenitaš A. Hypersaline waters in salterns—natural ecological niches for halophilic black yeasts. FEMS Microbiol Ecol 2000; 32:235-40; PMID:10858582.
12. Gostincar C, Grube M, Gunde-Cimerman N. Evolution of fungal pathogens in domestic environments? Fungal Biol 2011; 115:1008-18; PMID:21944213; http://dx.doi.org/10.1016/j.funbio.2011.03.004.
13. Prista C, Soeiro A, Vesely P, Almagro A, Ramos J, Loureiro-Dias MC. Genes from *Debaryomyces hansenii* increase salt tolerance in *Saccharomyces cerevisiae* W303. FEMS Yeast Res 2002; 2:151-7; PMID:12702302.
14. Gostincar C, Gunde-Cimerman N, Turk M. Genetic resources of extremotolerant fungi: a method for identification of genes conferring stress tolerance. Bioresour Technol 2012; 111:360-7; PMID:22386631; http://dx.doi.org/10.1016/j.biortech.2012.02.039.
15. Daran JM, Bell W, François J. Physiological and morphological effects of genetic alterations leading to a reduced synthesis of UDP-glucose in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 1997; 153:89-96; PMID:9252577; http://dx.doi.org/10.1111/j.1574-6968.1997.tb10468.x.
16. Dey NB, Bounelis P, Fritz TA, Bedwell DM, Marchase RB. The glycosylation of phosphoglucomutase is modulated by carbon source and heat shock in *Saccharomyces cerevisiae*. J Biol Chem 1994; 269:27143-8; PMID:7929458.
17. Boles E, Liebetrau W, Hofmann M, Zimmermann FK. A family of hexosephosphate mutases in *Saccharomyces cerevisiae*. Eur J Biochem 1994; 220:83-96; PMID:8119301; http://dx.doi.org/10.1111/j.1432-033.1994.tb18601.x.
18. Masuda CA, Xavier MA, Mattos KA, Galina A, Montero-Lomeli M. Phosphoglucomutase is an in vivo lithium target in yeast. J Biol Chem 2001; 276:37794-801; PMID:11500487.
19. Schade B, Jansen G, Whiteway M, Entian KD, Thomas DY. Cold adaptation in budding yeast. Mol Biol Cell 2004; 15:5492-502; PMID:15483057; http://dx.doi.org/10.1091/mbc.E04-03-0167.
20. Herscovics A, Orlean P. Glycoprotein biosynthesis in yeast. FASEB J 1993; 7:540-50; PMID:8472892.
21. Kepes F, Schekman R. The yeast SEC53 gene encodes phosphomannomutase. J Biol Chem 1988; 263:9155-61; PMID:3288631.
22. Do CB, Mahabhashyam MSP, Brudno M, Batzoglou S. ProbCons: Probabilistic consistency-based multiple sequence alignment. Genome Res 2005; 15:330-40; PMID:15687296; http://dx.doi.org/10.1101/gr.2821705.
23. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 2010; 59:307-21; PMID:20525638; http://dx.doi.org/10.1093/sysbio/syq010.