2019

Laboratory of Applied Stress Microbiology

Professor Hiroshi Takagi, Ph.D.

Originality !! *Serendipity !!*

The Takagi Laboratory

Professor: **Hiroshi Takagi**
(*1982: Ajinomoto → 1995: Fukui Pref. Univ. → 2006: NAIST*)

Associate Prof.: **Yukio Kimata**

Assistant Prof.: **Daisuke Watanabe, Ryo Nasuno**

Lab Assistant: Yoko Torisawa

8 Overseas students !!

5 Postdocs, 1 Technician, 22 Students (DC 7/MC 15)

Microbial Cells

Applied Molecular Microbiology

Microbial ***Bioscience***

Screening (mutants, isolates)
Genomic information

Biochemistry & Physiology
Molecular Genetics & Cell Biology

Analysis and improvement of microbial functions

Gene engineering
Metabolic engineering

Protein engineering
Cell engineering

- Construction of useful microorganisms
- Production of valuable substances
- Development of promising technology

Food
Life

Environment
Energy

Application to ***Biotechnology***

Applied Stress Microbiology

Hiroshi Takagi

Daisuke Watanabe

Ryo Nasuno

Stress response and adaptation mechanisms in yeast

1) Proline 2) Mpr1 (Arginine) 3) Nitric oxide 4) Ubiquitin system 5) Rim15

＜Keywords＞ oxidative stress, ROS, proline, transporter, mitochondria, arginine, NO, NO synthase, *S*-nitrosylation, redox regulation, ubiquitination, permease, phospholylation, transcription factor (Msn2, Pog1) ion stress, signal transduction *etc*.

L-Cysteine metabolism and its role in Escherichia coli

＜Keywords＞ cysteine, transporter, redox regulation, thiosulfate pathway *etc*.

Yeast is an important microorganism as …

Model for "Higher Eukaryotes"

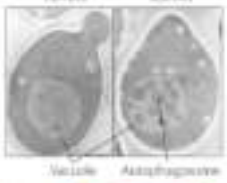
Autophagy

Novel Prize winner Dr. Y. Ohsumi

Awamori

Alcohol beverage brewed by my Yeast

Good "Ethanol Producer"



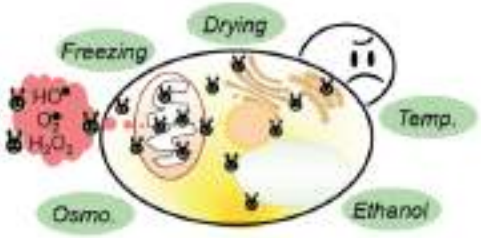
Industrial ***yeast breeding***

1) Improvement of cellular physiology
2) Improvement of yield or productivity
3) Extension of substrate range
4) Reduction of by-product formation
5) Pathways leading to new products

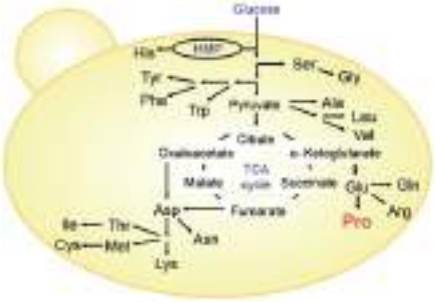
Improvement of fermentation ability

Diversity of product taste and flavor

Stress tolerance

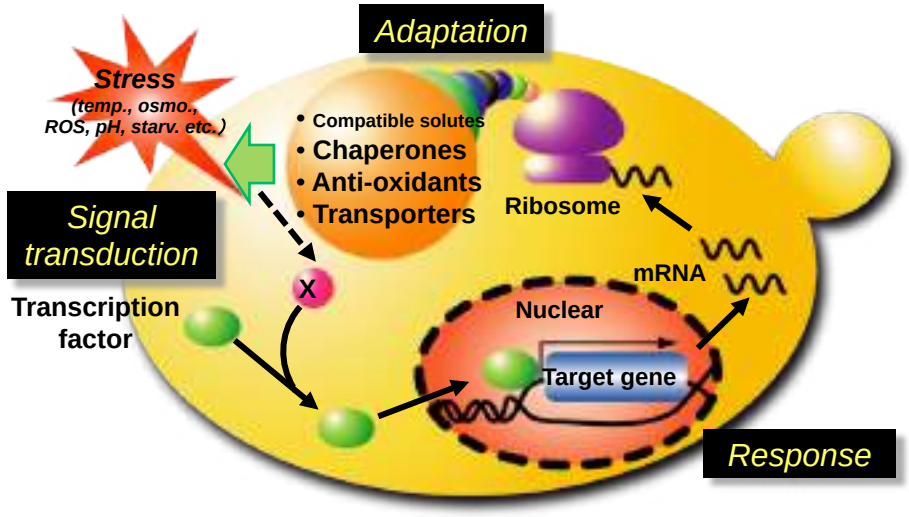


Metabolism modification

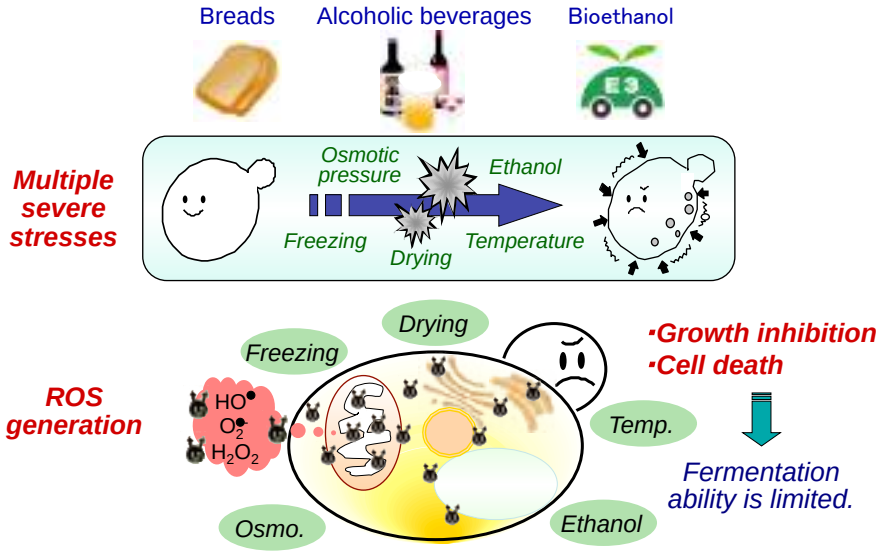


The budding yeast *Saccharomyces cerevisiae*

Cellular response and adaptation to environmental stresses



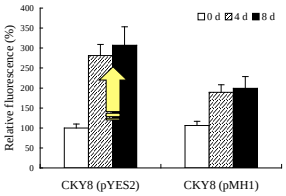
Industrial yeast strains



Stress tolerance is the key for yeast cells.

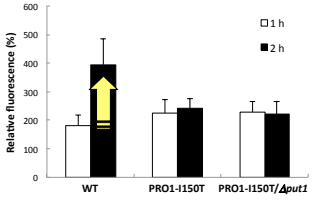
Yeast cells are exposed to oxidative stress.

Freezing (-20℃, 8 d)



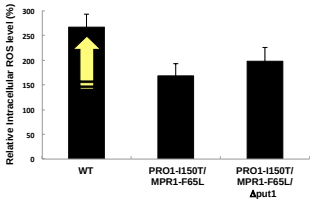
(Du & Takagi, ***J. Biochem.***, 2005)

Sucrose (30%, 2 h)



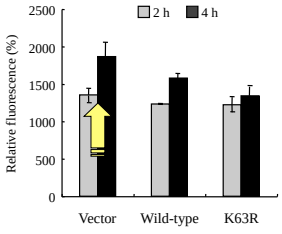
(Sasano *et al.*, ***Int. J. Food Microbiol.***, 2012)

Drying (42℃, 90 min)



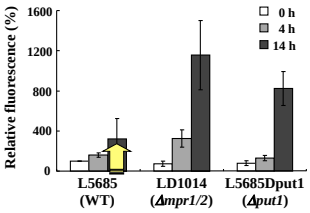
(Sasano *et al.*, ***Microb. Cell Fact.***, 2012)

Ethanol (18%, 4 h)



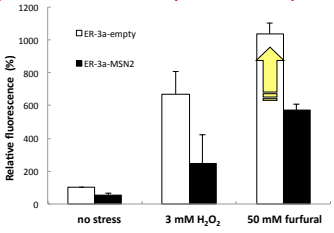
(Iinoya *et al.*, ***Biotechnol. Bioeng.***, 2009)

Temperature (39℃, 14 h)



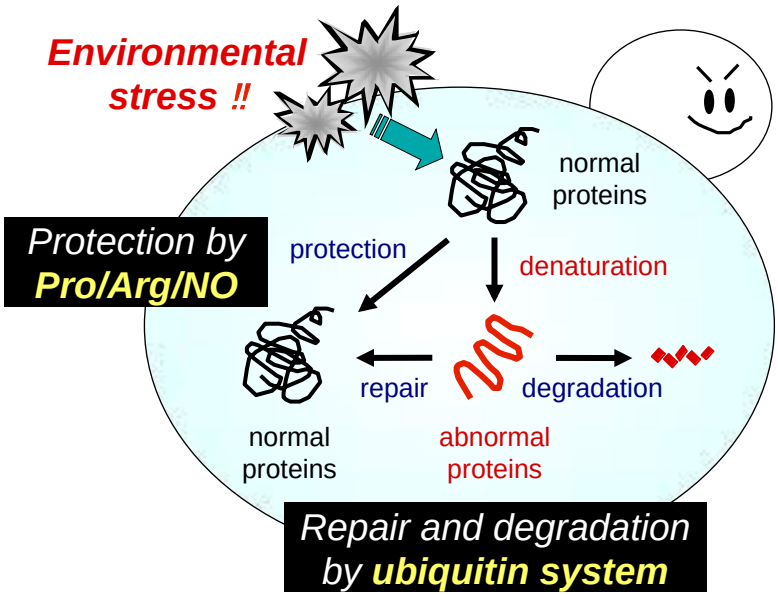
(Nishimura *et al.*, ***FEMS Yeast Res.***, 2010)

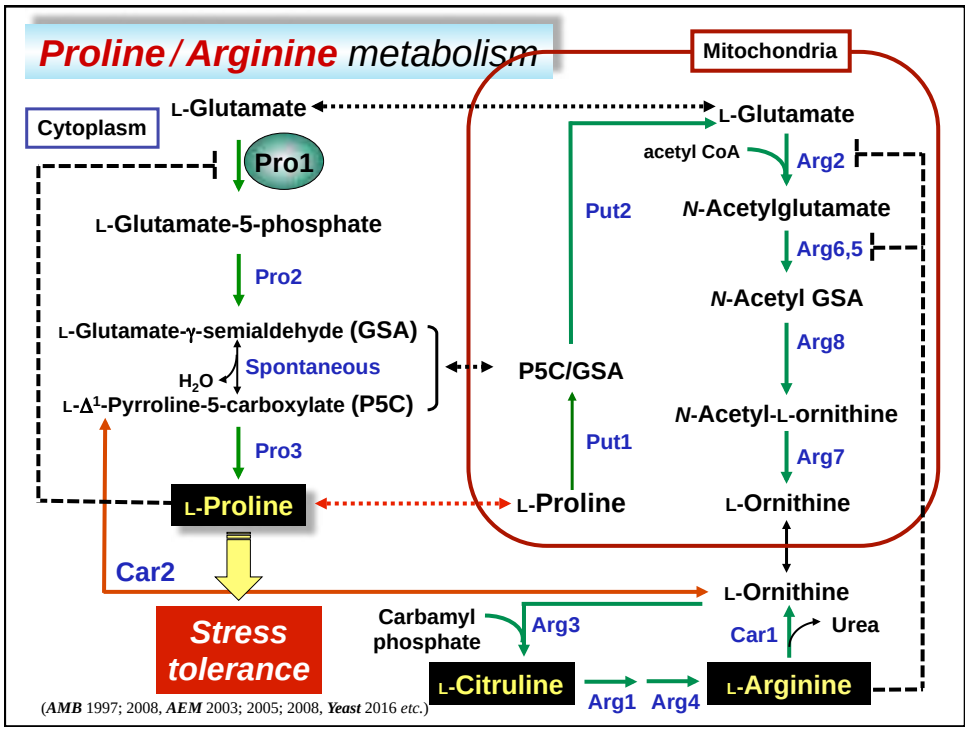
Furfural (50mM, 1 h)

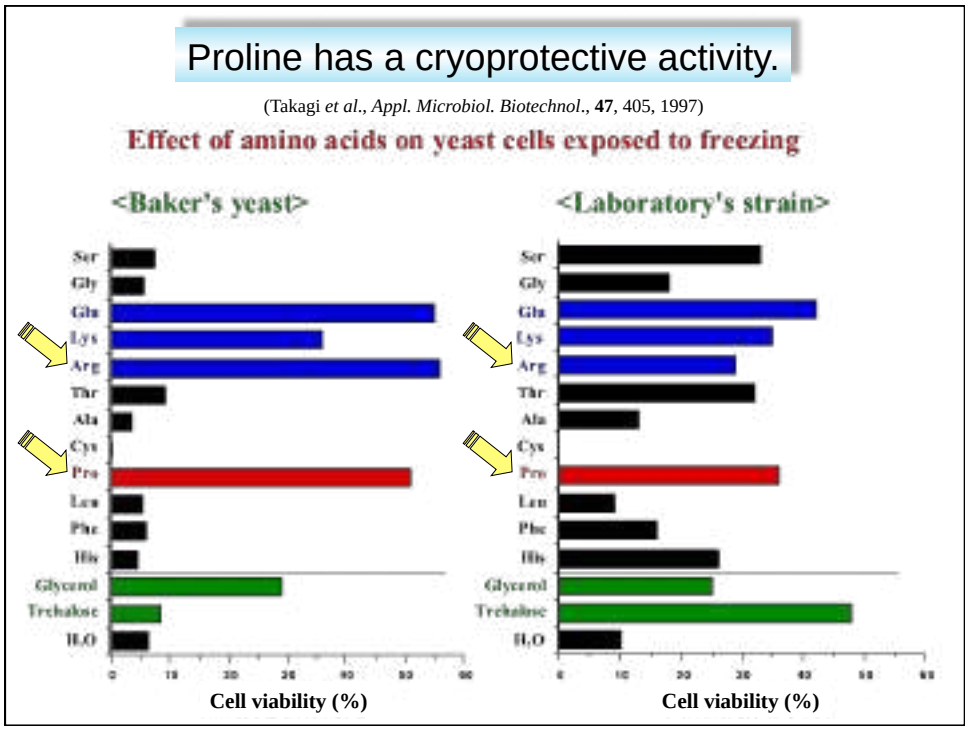


(Sasano *et al.*, ***J. Biosci. Bioeng.***, 2012)

Novel stress-tolerant mechanisms in yeast



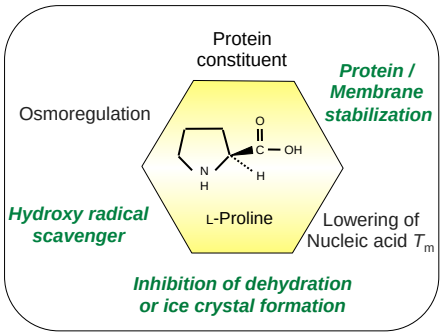

Proline / Arginine metabolism
Mitochondria
Cytoplasm
L-Glutamate
L-Glutamate
Pro1
acetyl CoA
Arg2
Put2
N-Acetylglutamate
L-Glutamate-5-phosphate
Arg6,5
Pro2
N-Acetyl GSA
L-Glutamate-γ-semialdehyde (GSA)
Arg8
H2O
Spontaneous
P5C/GSA
L-Δ1-Pyrroline-5-carboxylate (P5C)
N-Acetyl-L-ornithine
Put1
Arg7
Pro3
L-Proline
L-Proline
L-Ornithine
Car2
L-Ornithine
Carbamyl phosphate
Arg3
Car1
Urea
Stress tolerance
L-Citruline
Arg1
Arg4
L-Arginine
(AMB 1997; 2008, AEM 2003; 2005; 2008, Yeast 2016 etc.)


Proline has a cryoprotective activity.
(Takagi et al., Appl. Microbiol. Biotechnol., 47, 405, 1997)
Effect of amino acids on yeast cells exposed to freezing
<Baker's yeast>
<Laboratory's strain>
Ser
Gly
Glu
Lys
Arg
Thr
Ala
Cys
Pro
Leu
Phe
His
Glycerol
Trehalose
H2O
Cell viability (%)
Cell viability (%)

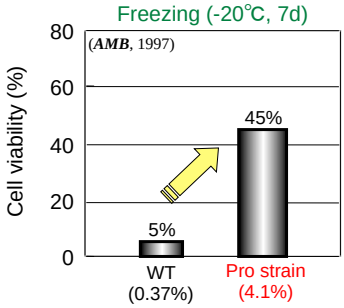
Proline *confers stress tolerance on yeast cells.*

◆In response to osmotic stresses, many bacterial and plant cells accumulate proline.
◆Yeast cells induce glycerol or trehalose synthesis, but do NOT increase proline level.

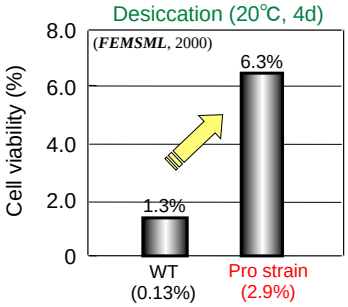
Physiological functions



Freezing (-20℃, 7d)



Desiccation (20℃, 4d)



Proline-accumulating mutants were isolated among proline analogue-resistant mutants.

① Proline

Appl. Microbiol. Biotechnol., **47**, 405, 1997; **79**, 273, 2008, **81**, 211, 2008; *FEMS Microbiol. Lett.*, **184**, 103, 2000; *Appl. Environ. Microbiol.*, **69**, 212, 2003; **69**, 6527, 2003; **71**, 8656, 2005; **73**, 4011, 2007; **74**, 5845, 2008; *J. Biosci. Bioeng.*, **94**, 2002; **100**, 538, 2005; **103**, 277, 2007; **116**, 576, 2013; *Biosci. Biotech. Biochem.*, **73**, 2131, 2009; **76**, 454, 2012; *Int. J. Food Microbiol.*, **152**, 40, 2012; **238**, 233, 2016; *J. Gen. Appl. Microbiol.*, **62**, 132, 2016; *Yeast*, **33**, 353, 2016; *FEBS Lett.*, **590**, 2906, 2016; *Microbial Cell*, **3**, 522, 2016.

< So far >

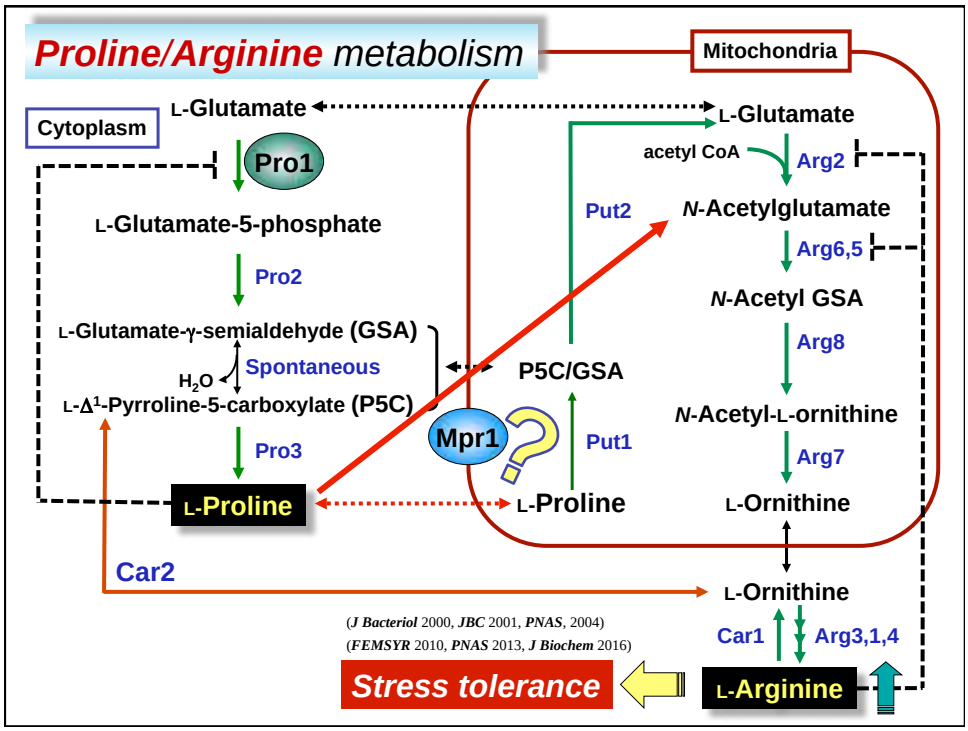
★ Proline protects yeast cells from various stresses as a ROS scavenger *!!*

★ Desensitization of feedback inhibition of γ-glutamyl kinase (Pro1) enhances proline accumulation and stress tolerance *!!*

< Current projects >

- Novel physiological functions (ribosomal autophagy, life span)
- Transport to mitochondria/vacuole
- Functional analysis of GK and PO

Breeding of novel stress-tolerant yeast strains with Pro engineering



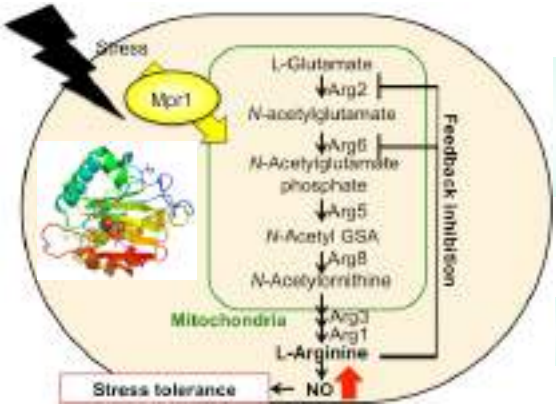
② Mpr1 (Arginine)

J. Bacteriol., **182**, 4249, 2000; *J. Biol. Chem.*, **276**, 41998, 2001; *Yeast*, **19**, 1437, 2002; **26**, 587, 2009; *J. Biochem.*, **133**, 67, 2003; **138**, 391, 2005; *Proc. Natl. Acad. Sci. USA*, **101**, 12616, 2004; **110**, 11821, 2013; *Appl. Microbiol. Biotechnol.*, **75**, 1343, 2007; **97**, 247, 2013; *FEMS Yeast Res.*, **8**, 607, 2008; *Biotechnol. Bioeng.*, **103**, 341, 2009; *Int. J. Food Microbiol.*, **138**, 181, 2010; *J. Biosci. Bioeng.*, **114**, 160, 2012; *J. Biochem.*, **159**, 271, 2016; *J. Ind. Microbiol. Biotechnol.*, doi: 10.1007/s10295-019-02177-3 *etc.*

< So far >

★ Mpr1 catalyzes *N*-acetylation of proline and its analogues (AZC, CHOP) *!!*

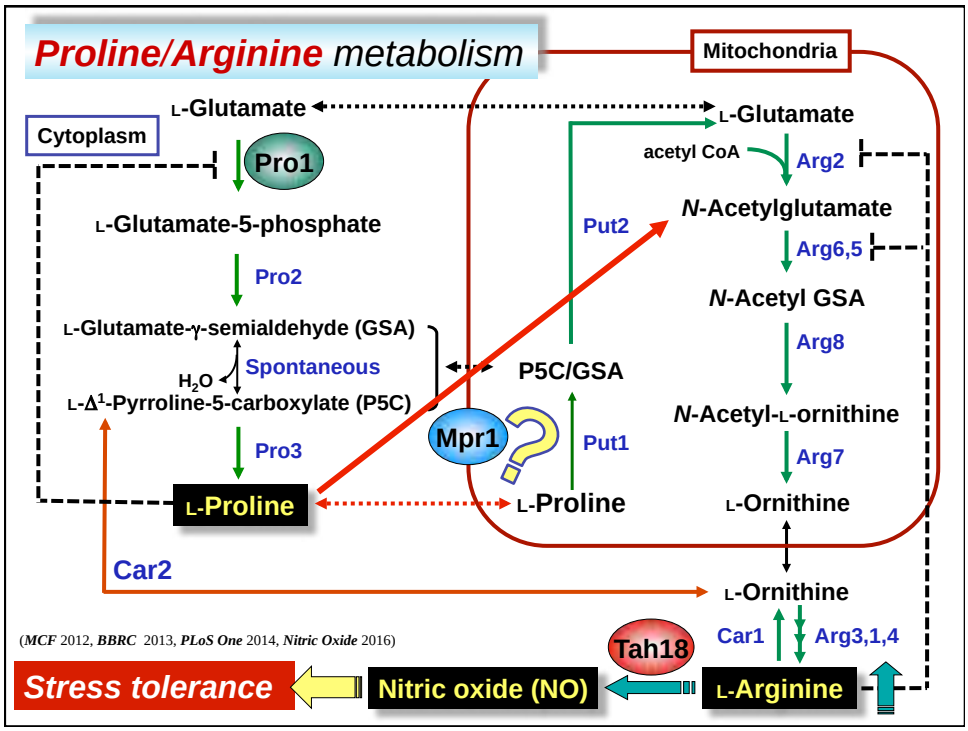
★ Mpr1 confers tolerance to oxidative stress on yeast cells by mediating arginine synthesis from proline *!!*

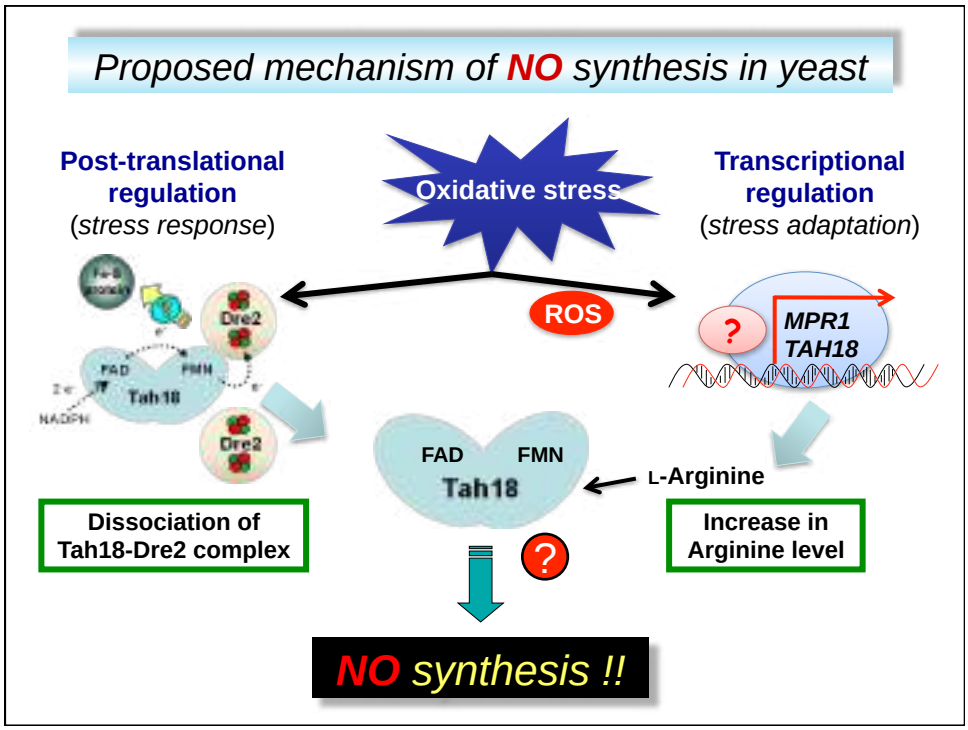


< Current projects >

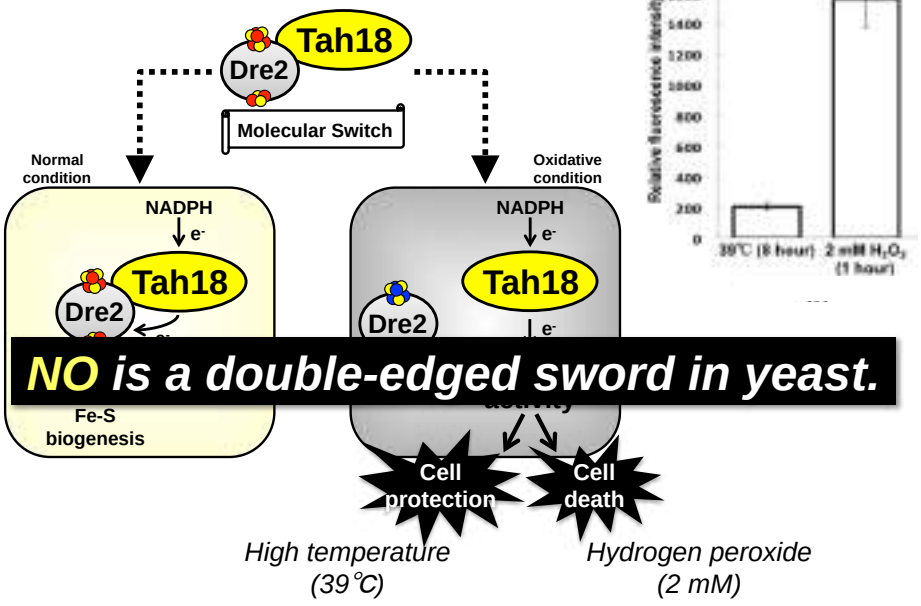
- Physiological functions (cellular substrate, Arg metabolism)
- Structural-based molecular design (higher activity and stability)

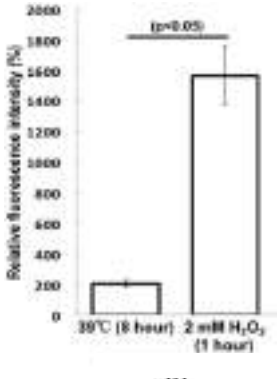
Breeding of novel stress-tolerant yeast strains by Mpr1 engineering


Proline/Arginine metabolism
Mitochondria
Cytoplasm
L-Glutamate
L-Glutamate
Pro1
acetyl CoA
Arg2
L-Glutamate-5-phosphate
Put2
N-Acetylglutamate
Arg6,5
Pro2
N-Acetyl GSA
L-Glutamate-γ-semialdehyde (GSA)
Arg8
H2O
Spontaneous
P5C/GSA
L-Δ1-Pyrroline-5-carboxylate (P5C)
N-Acetyl-L-ornithine
Mpr1
Put1
Arg7
Pro3
L-Proline
L-Proline
L-Ornithine
Car2
L-Ornithine
(MCF 2012, BBRC 2013, PLoS One 2014, Nitric Oxide 2016)
Tah18
Car1
Arg3,1,4
Stress tolerance
Nitric oxide (NO)
L-Arginine


Proposed mechanism of NO synthesis in yeast
Post-translational regulation
(stress response)
Oxidative stress
Transcriptional regulation
(stress adaptation)
ROS
Dre2
FAD
FMN
Tah18
NADPH
Dre2
?
MPR1
TAH18
FAD
FMN
Tah18
L-Arginine
Dissociation of Tah18-Dre2 complex
Increase in Arginine level
?
NO synthesis !!

Dual effects of NO in yeast





NO is a double-edged sword in yeast.

(Astuti *et al.*, ***Appl. Microbiol. Biotechnol.***, **100**, 9483, 2016)

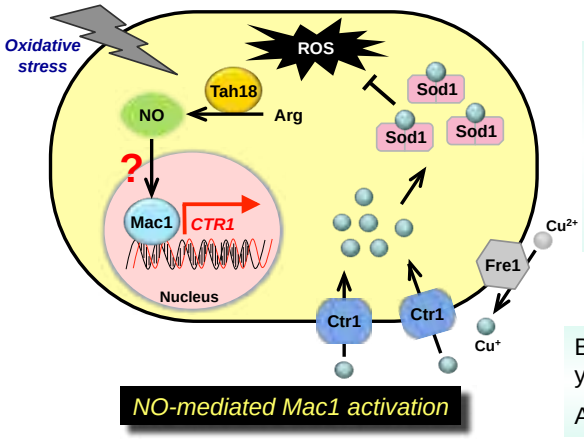
③ Nitric oxide

FEMS Yeast Res., **10**, 687, 2010; *Microb. Cell Fact.*, **11**:40 doi:10.1186/1475-2859-11-40, 2012: *Proc. Natl. Acad. Sci. USA*, **110**, 11821, 2013; *Biochem. Biophys. Res. Commun.*, **430**, 137, 2013; *PLoS One*, **9**, e113788, 2014; *Nitric Oxide-Biol. Chem.*, **52**, 29, 2016; **57**, 85, 2016; *Appl. Microbiol. Biotechnol.*, **100**, 9483, 2016; *Med. Mycol.* **59**, E63-E70, 2018.

< So far >

★ NO is produced from Arg through the Tah18-dependent activity *!!*

★ NO confers oxidative stress tolerance on yeast cells by enhancing Sod1 activity through the activation of Mac1 *!!*



NO-mediated Mac1 activation

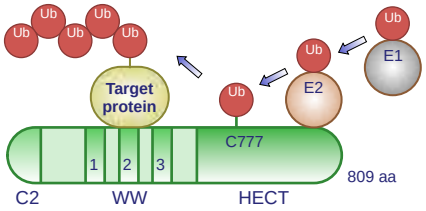
< Current projects >

- Regulation of synthesis (Tah18-Dre2 interaction)
- Physiological roles (cell protection vs. cell death)
- Protein modifications (*S*-nitrosylation, nitration)

Breeding of novel stress-tolerant yeast strains with NO engineering

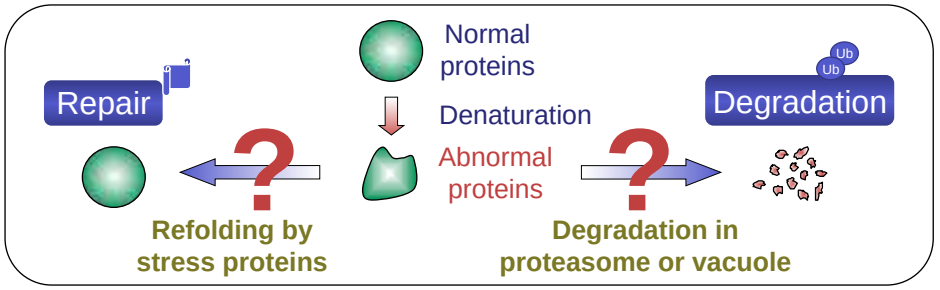
Application to pathogenic yeasts

Rsp5 is an essential E3 ubiquitin ligase.



Rsp5 participates in many events through ubiquitination of target proteins;

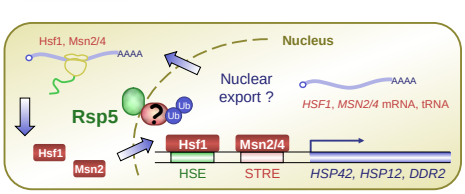
endocytosis of plasma membrane permeases, mitochondrial inheritance, degradation of the large subunit of RNA pol. II, biosynthesis of unsaturated fatty acids, actin cytoskelton organization, sporulation, ER-associated degradation etc.

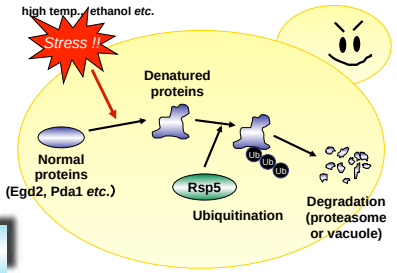


Rsp5 may be involved in repair / degradation of abnormal proteins.

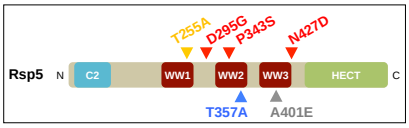
Rsp5

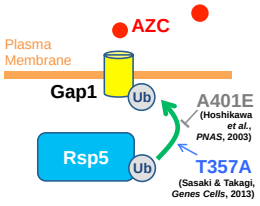
Involved in repair / degradation of abnormal proteins

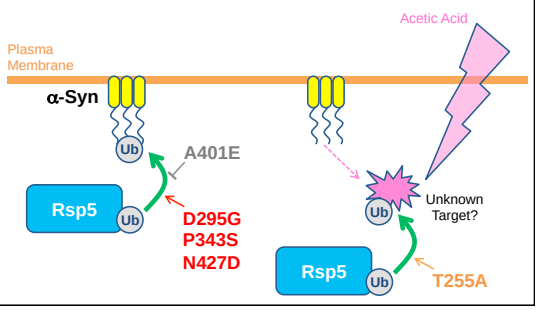




Required for degradation of Gap1







④ Ubiquitin system

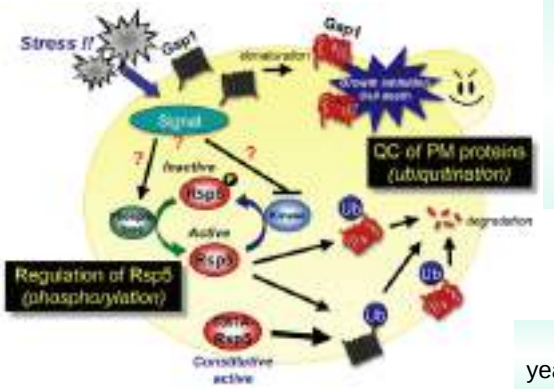
Proc. Natl. Acad. Sci. USA, **100**, 11505, 2003; *FEBS Lett.*, **580**, 3433, 2006; *Biosci. Biotech. Biochem.*, **70**, 2762, 2006; **73**, 2268, 2009; *FEMS Microbiol. Lett.*, **277**, 70, 2007; *Genes Cells*, **13**, 105, 2008; *FEMS Yeast Res.*, **9**, 73, 2009; **14**, 567, 2014; **17**, fox083, 2017; *FEBS J.*, **276**, 5287, 2009 ; *J. Brew. Distill.*, **3**, 1, 2012, *Genes Cells*, **18**, 459, 2013; *Eukaryot. Cell*, **13**, 1191, 2014; *J. Biochem.*, **157**, 251, 2015; *Appl. Environ. Microbiol.*, **84**, e00406-18, 2018.

＜ So far ＞

★ Rsp5 is involved in quality control of plasma membrane proteins *!!*

★ Rsp5 activity is regulated by phosphorylation of a conserved Thr357 *!!*

＜ Current projects ＞



- Recognition and degradation of abnormal proteins by Rsp5
- Functional improvement of Ub-system (Rsp5)
- Regulation of the Rsp5 activity via phosphorylation

Breeding of novel stress-tolerant yeast strains with improved Ub-system

Contribution to biotechnology

Stress-tolerant industrial yeasts

＜Expansion of **yeast**-related industry＞

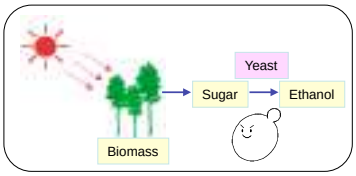
- Improvement of fermentation ability

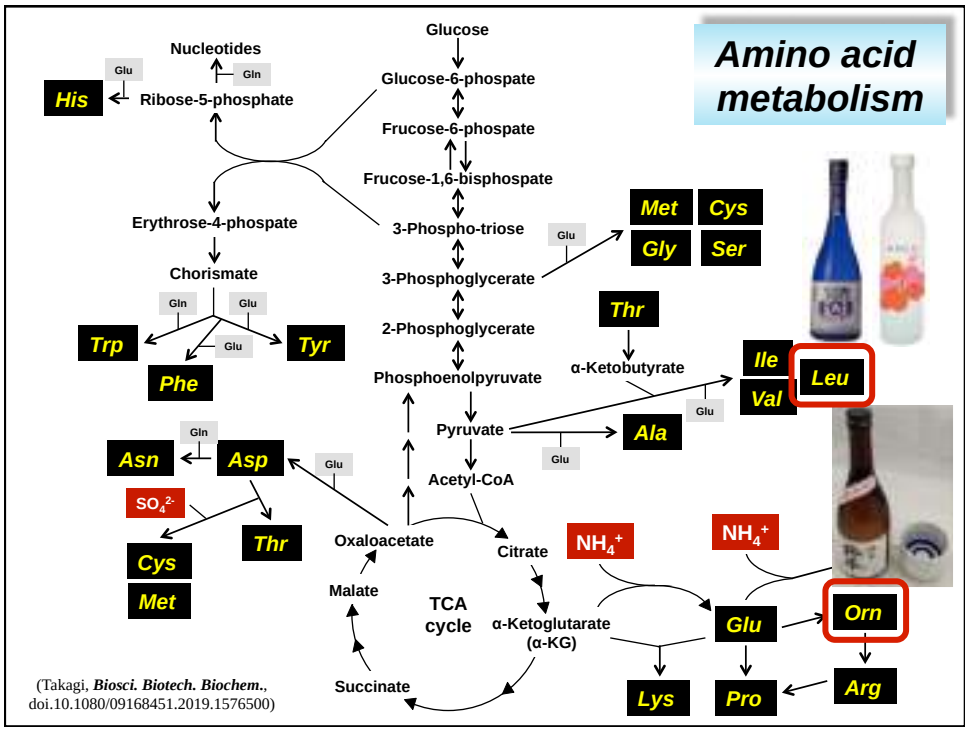
Efficient production of alcoholic beverages and breads

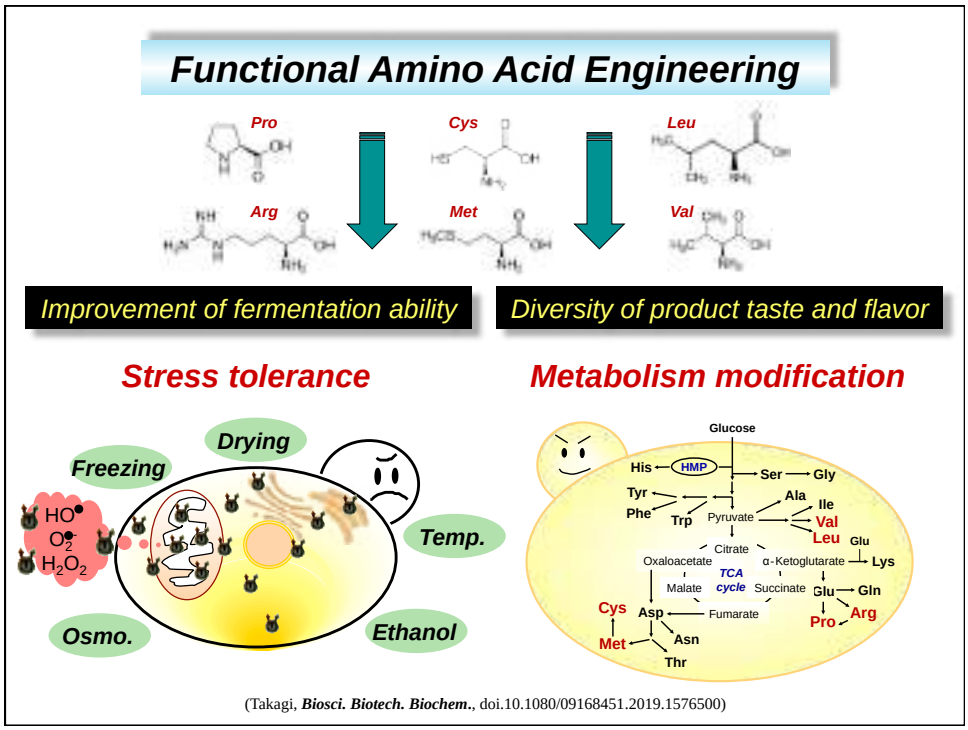
＜Creation of **yeast**-based new industry＞

- Improvement of compound productivity

High production of bioethanol, amino acid and protein




Amino acid metabolism
Glucose
Glucose-6-phospate
Frucose-6-phospate
Frucose-1,6-bisphospate
3-Phospho-triose
3-Phosphoglycerate
2-Phosphoglycerate
Phosphoenolpyruvate
Pyruvate
Acetyl-CoA
Oxaloacetate
Citrate
α-Ketoglutarate (α-KG)
Succinate
Malate
TCA cycle
Nucleotides
Ribose-5-phosphate
Erythrose-4-phospate
Chorismate
α-Ketobutyrate
His
Trp
Phe
Tyr
Asn
Asp
Thr
Cys
Met
Ala
Gly
Ser
Ile
Val
Leu
Lys
Pro
Arg
Orn
Glu
Gln
SO_4^{2-}
NH_4^+
(Takagi, Biosci. Biotech. Biochem., doi.10.1080/09168451.2019.1576500)

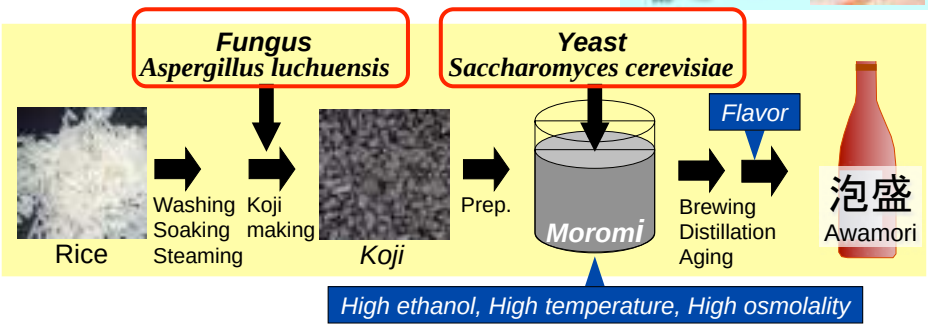

Functional Amino Acid Engineering
Pro
Cys
Leu
Arg
Met
Val
Improvement of fermentation ability
Diversity of product taste and flavor
Stress tolerance
Metabolism modification
Drying
Freezing
Temp.
Osmo.
Ethanol
HO•
O_2^-
H_2O_2
Glucose
HMP
His
Ser
Gly
Tyr
Phe
Trp
Pyruvate
Ala
Ile
Val
Leu
Glu
Lys
Citrate
Oxaloacetate
α-Ketoglutarate
Malate
TCA cycle
Succinate
Gln
Pro
Arg
Cys
Asp
Fumarate
Met
Asn
Thr
(Takagi, Biosci. Biotech. Biochem., doi.10.1080/09168451.2019.1576500)

What is **Awamori** ?

One type of *shochu*, a traditional distilled alcoholic beverage made from steamed rice in Okinawa, Japan.

Okinawa

Nara



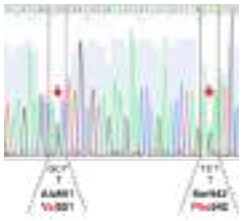
Proline / Arginine: Enhance stress tolerance of *awamori yeasts ?*

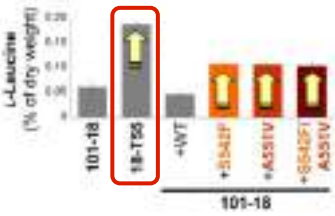
Leucine / Pheylalanine: Increase flavor components of *awamori ?*

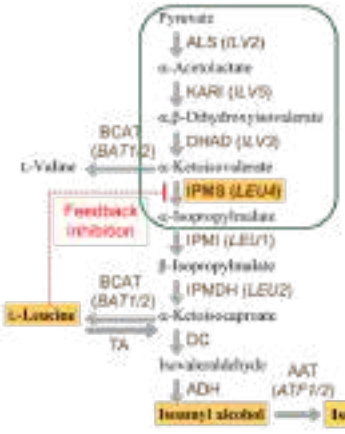
Awamori yeasts *that overproduce* ***leucine/isoamyl acetate***

(Takagi *et al.*, ***J. Biosci. Bioeng.***, **119**, 140, 2015)

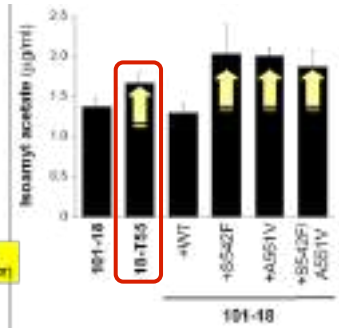
JBB Paper Award !!



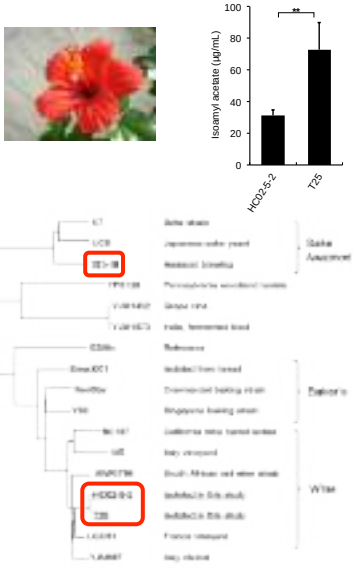
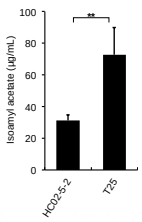
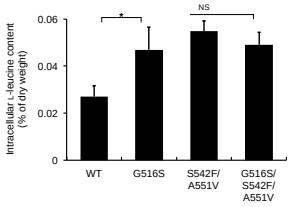
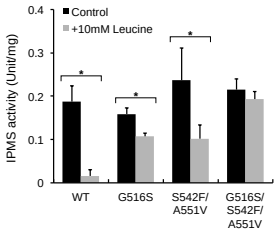




Ser542Phe/Ala551Val



atmospheric/vacuum distillation 50%/50% ***alcohol 35% 720ml ¥2,000***

A new Saccharomyces cerevisiae isolated from hibiscus flower and its mutant with leucine accumulation for Awamori brewing

(Abe *et al.*, ***Front. Genet.***, **10**, 490, 2019)