Mechanisms of Cellular Senescence and Longevity in *Saccharomyces cerevisiae*

A Doctoral Thesis

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Contents

Mechanisms of Cellular Senescence and Longevity in *Saccharomyces cerevisiae*

（出芽酵母の細胞老化と寿命決定機構に関する研究）

Chapter 1

General introduction

Lifespan is generally considered an important indicator for quality of life. Everyone might concern their own lifespan and care to slow aging for the quality of their life. As accompanied with development of medical care and improvement of nutrition, the average human lifespan is extending year after year; especially the Japanese life expectancy at birth is one of the longest in the world (women; 87.0 at rank 1, men; 80.0 at rank 8 in 2012) (1). Recently, further challenges to extend healthspan, rather than lifespan, by delaying senescence would be expected.

1.1 Genetic and environmental determinants of lifespan

Lifespan is thought to be largely determined by the combined effects of genetics and environmental factors (Figure 1.1). Many genes involved in aging have been identified in a variety of organisms, including mice, nematodes, and yeasts (2-4). Premature aging disorders, like Werner syndrome, Bloom's syndrome, and Hutchinson–Gilford Progeria Syndrome (HGPS), have been the subjects of immense interest as they recapitulate many of the phenotypes observed in physiological aging (5). They arise from mutations of a single gene. For example, the most common cause of HGPS is a single-letter "misspelling" in a gene on chromosome 1 that codes for lamin A, a protein that is a key component of the membrane surrounding the cell's nucleus (6).

One of the most important environmental factors is lifestyle, such as calorie intake. Oxidative stress is worldwide recognized as a fundamental component of the aging. Ultraviolet B-induced DNA damage, when left unrepaired, leads to accumulation of mutations, predisposing people to carcinogenesis as well as to premature aging (7). In response to many different environmental and physiological signals, nutrient and stress sensors modulate lifespan. Reduced activity of nutrient-sensing pathways or dietary restriction slows aging and increases lifespan (8). The nutrient-sensing pathways include the kinase target of rapamycin (TOR), AMP-activated protein kinase (AMPK), sirtuins, and insulin/insulin-like growth factor (IGF-1) signaling, among others (9), and these

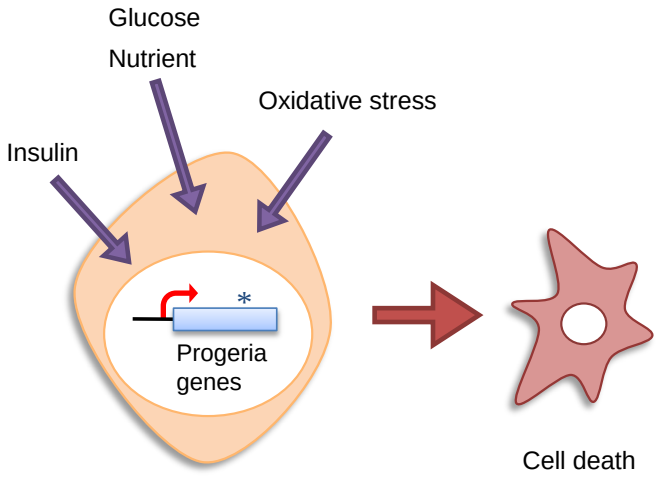

Figure 1.1 Cellular lifespan is determined by the combined effects of genetics and environmental factors. For genetic determinants, progeria genes are well studied. For environmental determinants, nutrient (including glucose), insulin, and oxidative stress modulate lifespan. The nutrient and stress sensors are also involved in aging.

nutrient signaling molecules also regulate stress response pathways (10).

1.2 Cellular senescence in mammals

Senescence is the biological process of cellular and organismal changes that deteriorate physiological function with the passage of time, resulting eventually in death. Accelerated cellular senescence and progressed organ dysfunction lead to a gradual decline in the physical and mental faculties of individuals (11). This deterioration is believed to be due to cumulative damage to molecular and cellular structures, and by programmed alteration of gene expression. Human somatic cells have a limited capacity to divide in culture and eventually enter replicative senescence, a state of irreversible proliferation arrest (Figure 1.2A), leading to tissue dysfunction (12). Biological aging is the main risk factor for human pathologies, such as cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases (13), and removal of senescent cells can prevent or delay tissue dysfunction and extend the healthspan (14).

In mammals, senescent cells exhibit diverse alterations in their cellular and biochemical features: an enlarged and flattened cellular morphology, an increase in the production of reactive oxygen species (ROS), senescence-associated β-galactosidase

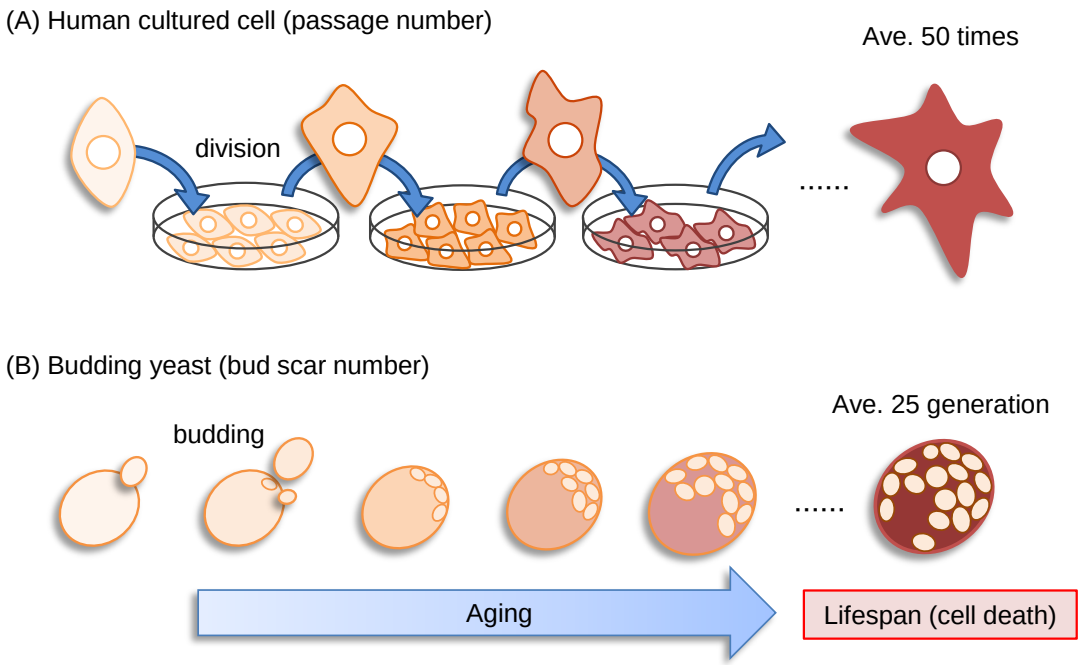

Figure 1.2 Budding yeast as a model for cellular senescence. (A) Cellular senescence of human cultured cells. The term passage number refers to the number of times that a cell population has been removed from the culture vessel and undergone a subculture (passage) process. (B) Cellular senescence of budding yeast. As cells undergo cell divisions, senescence proceeds.

(SA-β-gal) activity, and senescence-associated secretory phenotype (SASP) (13,15). Some of these age-related cellular phenomena are explained by time-series gene expression profiles; the senescent process is divided into four stages (early, middle, advanced, and very advanced), with specific genes being prominently expressed at each of these stages (16).

1.3 Budding yeast as a model organism in cellular aging research

The budding yeast, *Saccharomyces cerevisiae*, is a useful and powerful model for cellular aging research. Most benefits of using yeast are based on its short generation time, convenient and cheap experimental setups, straightforward genetic approaches (17). Furthermore, yeast has similarities (homologues and orthologues) with mammalian (including human) cells, making it good model for human diseases (18).

Yeast cells age as they undergo division, as do cultured mammalian cells: their size

increases, their shape is altered, their cell cycle slows down, and they become sterile (19). Furthermore, the nucleolus of aged yeast cells tends to be larger and/or more fragmented, and the mitochondria become dysfunctional (19,20). The rate of protein synthesis and ribosome activity decrease linearly with age (21). In mother cells during the budding process, oxidative stress, protein aggregation, and extrachromosomal rDNA circles (self-replicating circles of ribosomal DNA) accumulate and cause senescence (20). These accumulations are relieved by calorie restriction, which helps slow aging and extend the lifespan in yeast (22).

In the yeast, two types of cellular aging have been proposed: chronological and replicative (Figure 1.3). The chronological lifespan is the length of time a population remains viable in the post-diauxic and stationary phases (23). The replicative lifespan of a yeast cell is defined as the number of daughter cells that a mother cell can generate before dying (24). The budding yeast is a unique model for studies on the lifespan of a single cell, because its asymmetric division makes it easier to analyze a population of cells compared with symmetrically-dividing mammalian cells (Figure 1.2B). The median replicative lifespan of most wild-type strains is ~25 generations; the maximum recorded is ~40 generations (25). Similarly to mammals, calorie restriction extends the replicative lifespan in yeast. Calorie restriction caused a metabolic shift from fermentation to respiration, resulting in activation of Sir2p, a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase belonging to the sirtuin family that acts as a master regulator of anti-aging (26), or reduction of activity of the nutrient-responsive kinases Sch9p and TOR (27).

1.4 A metabolomic approach for lifespan study

Metabolomics is an established and useful tool in functional genomics. But, many reports have suggested that a combination of metabolomics and transcriptomics is required for the complete elucidation of gene function (28,29). Together with transcriptomics and proteomics, metabolomics is also applicable for elucidation of aging and lifespan.

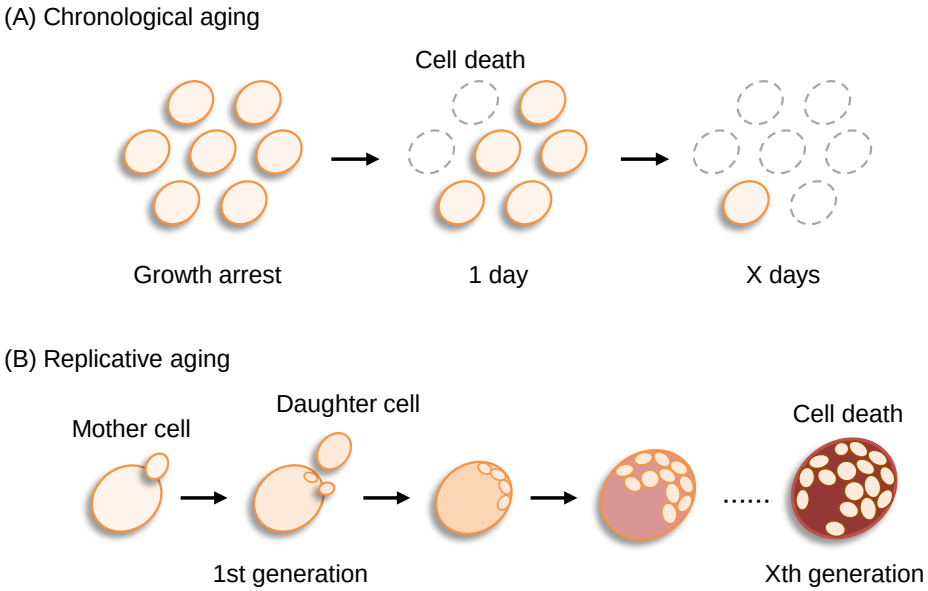

Figure 1.3 Model of two types of cellular aging in the yeast. (A) The chronological lifespan is the length of time a population remains viable in the non-dividing state. (B) The replicative lifespan is defined as the number of daughter cells that a mother cell can generate before dying.

In a previous study, fingerprinting of identified compounds using gas chromatography–mass spectrometry (GC-MS) and capillary electrophoresis-mass spectrometry (CE-MS) revealed a correlation between replicative lifespan and metabolic profile in *S. cerevisiae* (30). The levels of amino acids (e.g., glutamine and proline) and nucleotide derivatives (e.g., inosine and cAMP) correlated most closely with replicative lifespan. Furthermore, Yoshida *et al*. predicted replicative lifespan-related genes using multivariate analysis of the metabolome data and found novel genes that regulates replicative lifespan. These showed that longevity can be quantitatively expressed using the whole metabolic profile of the cell, while metabolomics was conventionally used only to discriminate among a few different cell types. However, it remains unclear that whether correlated metabolites is a cause of longevity and that how these genes regulates replicative lifespan.

1.5 Objectives of this study

In this thesis, what regulates replicative lifespan was investigated using the budding

yeast *S. cerevisiae* from the point of view of metabolism and gene expression in various mutants and during aging process. This Chapter 1 shows the background information for this study.

In Chapter 2, it was investigated that how γ-aminobutyric acid (GABA) metabolism pathway genes regulate replicative lifespan. Alteration of cellular metabolism expect for GABA contents seemed to cause lifespan regulation. In Chapter 3, to determine what change initiates the aging process, the early stage of replicative aging cells was focused. In the cells at about half the mean lifespan, amino acid biosynthesis declined, and sugar and tricarboxylic acid (TCA) cycle metabolism increased. In Chapter 4, whether vitamin B6 affects replicative lifespan was investigated. Vitamin B6 was shown to be important to replicative lifespan in yeast.

Chapter 2

Replicative lifespan regulation by GABA metabolism pathway genes

2.1 Introduction

It has been reported that many metabolism-related genes, including genes that encode metabolic transcription factors and enzymes, are involved in lifespan (4,31-33). Recently, Yoshida *et al*. revealed a correlation between replicative lifespan and metabolic profile of identified compounds using GC-MS and CE-MS in *S. cerevisiae* (30). The levels of amino acids (e.g., glutamine and proline) and nucleotide derivatives (e.g., inosine and cAMP) correlated most closely with replicative lifespan. Furthermore, they established a multivariate model to predict lifespan from a metabolic profile and demonstrated that three genes—utilization of GABA (*UGA*)*3*, five zinc fingers (*FZF*)*1*, and uridine hydrolase (*URH*)*1*—are aging-related genes because disruption of *UGA3*, *FZF1*, or *URH1* resulted in lifespan extension.

The *UGA3* gene encodes a zinc-finger transcription factor necessary for GABA-dependent induction of the *UGA* structural genes, *UGA1*, *UGA2*, and *UGA4* (34,35) (Figure 2.1). Uga1p (GABA transaminase) deaminates GABA to succinate semialdehyde. Uga2p (succinate semialdehyde dehydrogenase) converts succinate semialdehyde to succinic acid, which is supplied to TCA cycle. Uga4p (GABA permease) transports GABA to the vacuole. These *UGA* structural genes are involved in the use of GABA as a nitrogen source (34). Uga3p is required for transcriptional induction of the *UGA* structural genes in the presence of GABA and maintenance of basal transcription of the *UGA* genes in the absence of GABA (36).

To understand the cause of the replicative lifespan increase in the *UGA3* deletion mutant, deletions of each *UGA* structural gene that is under the control of Uga3p for effects on replicative lifespan were tested. Deletion of *UGA1* extended lifespan, but deletion of *UGA2* or *UGA4* did not. It was also found that *GAD1*, which encodes a GABA-metabolizing enzyme, was involved in aging. Respiration was not increased in

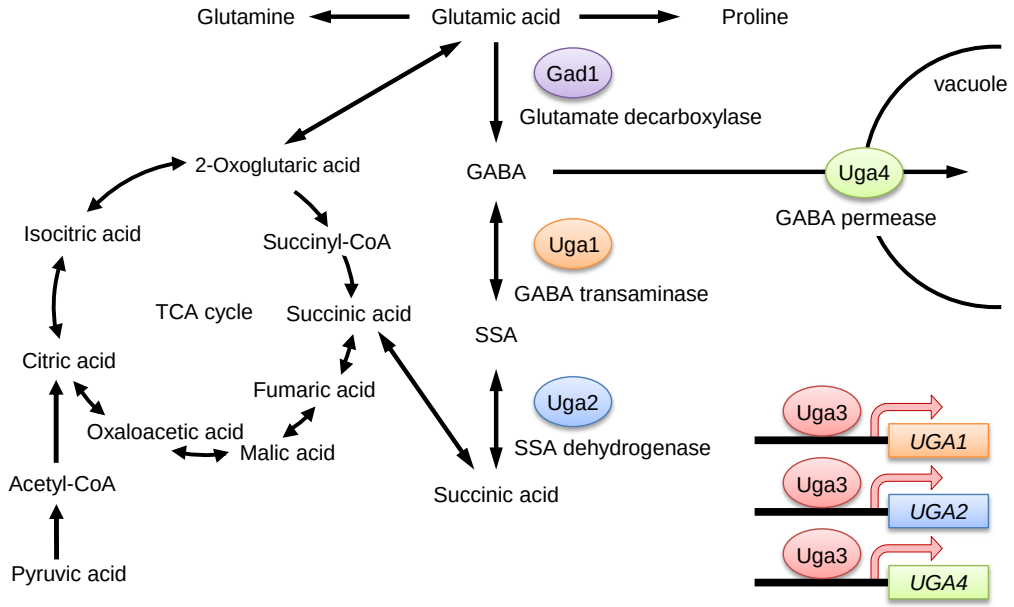

Figure 2.1 The synthesis, conservation and metabolism of GABA. Gad1p, glutamic acid decarboxylase; Uga1p, γ-aminobutyric acid (GABA) transaminase; Uga2p, succinic semialdehyde (SSA) dehydrogenase; Uga4p, GABA permease. Transcription factor Uga3p activates transcription of *UGA1*, *UGA2*, and *UGA4* genes.

the long-lived Δ*uga1* and Δ*gad1* cells. Deletion of *SIR2*, a yeast sirtuin, prevented lifespan extension by deletion of *UGA1* and *GAD1*. Based on these findings, the hypothesis that reduced activity of the GABA-metabolizing enzymes increases lifespan in a *SIR2*-dependent manner was discussed.

2.2 Materials and methods

2.2.1 Strains and medium

All *S. cerevisiae* strains used in this study were derived from BY4742 (*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*). Deletion strains were obtained from the MATα ORF deletion collection (Open Biosystems, USA). Yeast extract (1%), Bacto Peptone (2%), dextrose (2%) (YPD) medium was used for routine cultures.

2.2.2 Replicative lifespan determination

Replicative lifespan was assayed with minor modifications as described previously (37). Yeast cells were thawed from frozen stock and streaked onto YPD agar plates or

YPD plates containing 200 μg/mL G418. After 3 days, a single colony was transferred to 2 mL of YPD liquid media, and cells were grown overnight. The culture was diluted 1:500 in fresh YPD liquid medium, and a sample was spread onto YPD agar plate containing 10 μg/mL phloxine B. YPD agar plates containing GABA (0.1% or 0.5%) were used as indicated in the text. Using a micromanipulator, 48 cells were arrayed on a YPD plate and allowed to undergo 1 or 2 divisions. Virgin cells were then selected and subjected to lifespan analysis. Except during manipulation, plates were sealed with Parafilm and incubated at 30°C during the day and stored at 4°C at night to avoid excessive budding. Daughter cells were removed by gentle agitation with a dissecting needle and scored every 2 h. For each of the 48 cell lines, buds from each mother cell were counted until division of living cells ceased or cells were stained with phloxine B. This assay was performed at least twice with each strain. For statistical analysis, lifespan data sets were compared by a Wilcoxon rank-sum test. Two strains are stated to have a significant difference in lifespan when $P < 0.01$.

2.2.3 GC-MS analysis

Sample preparation from dried yeast cells and GC-MS analysis were performed as described previously (30). GC-MS analysis was performed independently 5 times for each strain. For comparison of intracellular levels of GABA, succinic acid, and glutamic acid, the area under the peak representing the compound was measured. To process GC data, raw chromatographic data (Pegasus file, *.peg) were converted into ANDI files (Analytical Data Interchange Protocol, *.cdf). The ANDI formatted data was converted and transferred between different mass spectral instruments. Baseline correction, peak detection, and peak alignment was performed with the free software MetAlign (Wageningen UR, The Netherlands, freely available at http://www.pri.wur.nl/UK/products/MetAlign/). The distinctive *m*/*z* peak for each compound was normalized on the basis of the intensity of the ribitol peak. Each metabolite was identified on the basis of an in-house chemical library. An *m*/*z* peak was selected as a distinctive *m*/*z* peak in the mass spectrogram if its intensity was not affected by neighboring peaks.

2.2.4 ^{1}H-nuclear magnetic resonance (NMR) analysis

Extraction of metabolites from yeast cells was performed as described previously (38). Yeast cell cultures (25 mL) were inoculated at 4.0×10^6 cells/mL and grown between 5–6 h to reach an OD of 1.0. The cells were isolated from culture medium by centrifugation (5 min; 1,750 × g), washed in water once. Then, 2 mL of 75% (v/v) ethanol solution, at 80°C, was added directly to the cell pellet. This mixture was mixed by vortex for 30 sec and heated for 3 min to 80°C. The mixture was cooled on ice for 3 min and subsequently dried in rotary vacuum concentrator at 35°C.

^{1}H-NMR analysis was performed as described previously (39). The dried extracts were dissolved in 0.6 mL of 0.1 M potassium phosphate buffer, pH 7.0, in D_2O, containing 1 mM sodium 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP), mixed by vortex, and then centrifuged at 16,000 × g for 10 min to remove material that had not dissolved. ^{1}H-NMR spectra were acquired at 400 MHz into 4,096 data points using a pulse angle of 90°, an acquisition time of 0.8 sec, and a sweep width of 5 kHz. The overall pulse repetition time was 5 sec. The samples were spun at 16 Hz and maintained at 30°C during data acquisition. The spectra were the sum of 128 transients. The chemical shift scale was referenced to the signal from TSP at 0.0 ppm.

Reduction of date from ^{1}H-NMR analysis was carried out by binning the spectra into 0.05 ppm regions; i.e., each spectrum was divided into 0.05 ppm regions, and the total signal within each region was integrated between 0.0 ppm and 10 ppm. The region from 4.4 ppm to 5.5 ppm, which contained the residual signal from the water resonance, was excluded. This resulted in each spectrum being reduced to a vector of length 176.

2.2.5 Multivariate analysis

The data sets from ^{1}H-NMR and GC-MS analyses were judged in all cases by orthogonal projection on latent structure-discriminant analysis (OPLS-DA) using SIMCA-P+ 12.0.1 (Umetrics, Sweden).

2.2.6 Fluorescence-based assay of cell respiration

Cellular oxygen consumption was assessed using the MitoXpress probe, according

to manufacturer's protocol (Luxcel Biosciences, Cork, Ireland) and as described previously (40). Cultured cells were diluted with medium to the desired concentration (OD=1.0), and 150 μL/well was dispensed into a 96-well plate (black body, flat bottom). An oxygen-sensitive probe supplied as dry powder in a vial, was reconstituted in 1 mL of water. Next, 10 μL of this solution was transferred into each well, and the wells were then covered with 100 μL of prewarmed (30°C) mineral oil to block ambient oxygen from the cells. Time-resolved measurements were carried out at 30°C on a Fusion α-FP Microplate Analyzer (PerkinElmer Life Sciences, Waltham, MA, USA) using 400-nm excitation and 645-nm emission filters, reading every 1.5 minutes for 120 min. Oxygen consumption rates were assessed by determining the rate of increase in the probe fluorescent signal for each sample. Experiments were performed in triplicate.

2.3 Results

2.3.1 The UGA1 gene encoding GABA transaminase regulates replicative lifespan

Deletion of the *UGA3* gene extends replicative lifespan; *UGA3* deletion mutant budding yeast cells produce about 60% more buds than wild-type cells (30). The *UGA3* gene encodes a zinc-finger transcription factor that is required for induction of *UGA1*, *UGA2*, and *UGA4* when GABA is present in culture medium (34,36). Even in the absence of GABA, deletion of *UGA3* down-regulates the basal transcription of *UGA1* (36). Therefore, it was expected that down-regulation of the Uga3p target genes caused increased lifespan.

To determine whether *UGA1*, *UGA2*, and/or *UGA4* were involved in aging, the replicative lifespan of each individual deletion mutant was measured (Figure 2.2A). Deletion of *UGA1* resulted in an increase in an average replicative lifespan of approximately 40% compared with the wild-type strain BY4742, while no increase in lifespan was observed for Δ*uga2* or Δ*uga4* mutants. Therefore, it was concluded that the *UGA1* gene, which encodes the GABA transaminase that deaminates GABA to succinate semialdehyde, negatively regulated replicative lifespan. These findings suggested that longevity of the Δ*uga3* mutant was caused by transcriptional down-regulation of *UGA1*, but not by changes in *UGA2* and *UGA4* regulation.

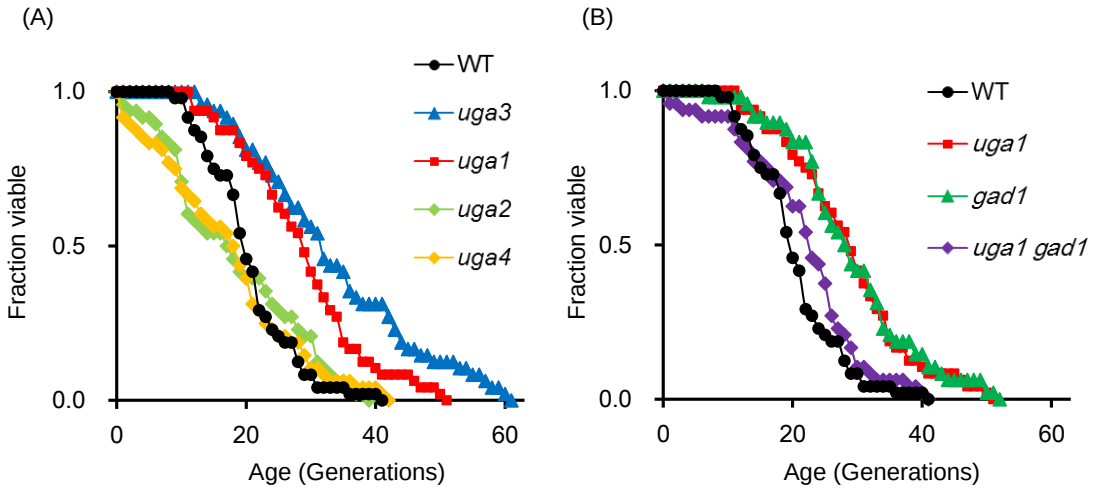

Figure 2.2 Replicative lifespans of cells carrying a deletion in *UGA1* or *GAD1*, genes encoding GABA-metabolizing enzymes. (A) Lifespan of deletion mutants of Uga3p target genes was determined. (B) Lifespan of the *GAD1* deletion mutant and the *UGA1 GAD1* double mutant was determined. Replicative lifespan was measured at least twice for each strain, and one measurement for each strain is shown. Average lifespans: BY4742 (wild type, WT), 20.7 generations; Δ*uga3*, 33.7; Δ*uga1*, 28.9; Δ*uga2*, 18.4; Δ*uga4*, 17.4; Δ*gad1*, 29.3; Δ*uga1* Δ*gad1*, 21.9.

2.3.2 The GAD1 gene encoding glutamate decarboxylase regulates replicative lifespan

Because Uga1p catalyzes deamination of GABA, another GABA-metabolizing enzyme gene (*GAD1*) that encodes glutamate decarboxylase, which converts glutamic acid into GABA (41) (Figure 2.1), was investigated. Deletion of *GAD1* resulted in an increase in average lifespan of approximately 40%, similar to that of *UGA3* and *UGA1* (Figure 2.2B). It was concluded that the *GAD1* gene also negatively regulated replicative lifespan. These results indicated that the GABA-metabolizing enzyme genes, *UGA1* and *GAD1*, are aging genes.

The increase in lifespan associated with the *UGA1* and *GAD1* deletion mutants led to the speculation that these deletions may additively increase lifespan. A Δ*uga1* Δ*gad1* double deletion mutant strain was constructed by tetrad dissection and determined its replicative lifespan. Contrary to the expectation, lifespan of the double mutant was shorter than that of the Δ*uga1* and Δ*gad1* single mutants and comparable to that of the wild-type strain (Figure 2.2B).

2.3.3 Supplementation of GABA to culture media has no effect on lifespan

Addition of 0.1% GABA to culture media induces transcription of the *UGA1*, *UGA2*, and *UGA4* genes (34,36), and replicative lifespan of the wild-type strain was measured on YPD plate medium supplemented with GABA. There was no significant difference between lifespan on GABA-containing and GABA-free media for wild-type cells. The mean lifespan on 0.1% GABA medium was 21.7 ± 8.9 generations and the maximal lifespan was 44 generations while the mean lifespan on YPD medium without GABA 22.4 ± 8.4 and the maximal lifespan 42. When GABA concentration was increased to 0.5%, the mean lifespan was 21.4 ± 7.2 generations and the maximal lifespan was 41 generations. Therefore, it was concluded that addition of GABA sufficient to activate transcription of the *UGA* structural genes had no effect on lifespan. Interestingly, increased transcription of the Uga3p target genes seemed not to affect lifespan, while deletion of *UGA3* did. This result was similar to the case of a *RAS1* deletion; *RAS1* encodes a GTPase involved in G-protein signaling in the adenylate cyclase activating pathway. A *RAS1* deletion increases lifespan, but overexpression of *RAS1* does not result in a change in lifespan (42). There is no report indicating that GABA supplements extend lifespan in any organism, including yeast.

2.3.4 The metabolomic profile of GABA-metabolizing enzyme mutants correlates with replicative lifespan

To investigate the reason for the increase in lifespan of Δ*uga1* and Δ*gad1* cells, the metabolites in the GABA metabolic pathway (GABA, glutamic acid, and succinic acid) in the long-lived (Δ*uga3*, Δ*uga1*, and Δ*gad1*) and normal-lived (Δ*uga2*, Δ*uga4*, Δ*uga1* Δ*gad1*, and wild type) strains were measured using GC-MS analysis. The intracellular GABA levels of all deletion mutants except for Δ*uga2* were comparable to that of the wild-type strain (Figure 2.3A). This result indicated that intracellular GABA levels did not regulate replicative lifespan. There was almost no difference in intracellular level of glutamic acid, the substrate of Gad1p, between strains; however, there was a slight decrease in Δ*uga3* single and Δ*uga1* Δ*gad1* double mutant cells (Figure 2.3B). This observation was consistent with the observation from previous study that glutamic acid

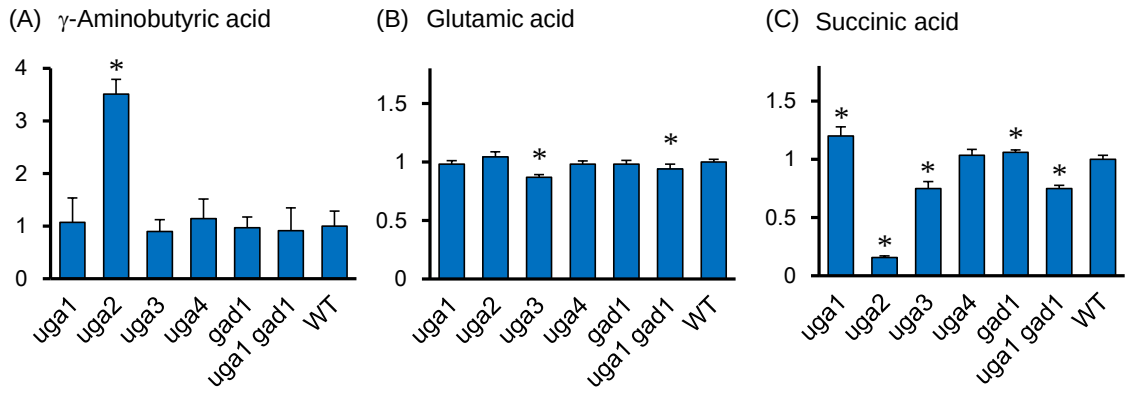

Figure 2.3 Comparison of intracellular levels of GABA metabolites in mutants carrying deletions of genes in the GABA metabolic pathway. (A) GABA. (B) glutamic acid. (C) succinic acid. Data are expressed as mean ± SEM. *P < 0.05 (n=5).

levels do not correlate with lifespan extension (30). Succinic acid levels were significantly lower in the *UGA2* deletion mutant. Interestingly, the level of succinic acid in the long-lived Δ*uga1* and Δ*gad1* mutants was elevated (Figure 2.3C), suggesting that succinic acid might be a key contributor to lifespan extension. Low levels of succinic acid in the long-lived Δ*uga3* mutant may be a consequence of down-regulating *UGA2* expression.

Analysis of levels of the metabolites in the GABA metabolic pathway did not reveal a striking correlation between longevity and metabolite levels in the *UGA* mutants; therefore, the whole-cell metabolite levels were surveyed by ^{1}H-NMR-based metabolomic analysis. The cell extracts were prepared from mid-logarithmic phase yeast liquid cultures of the long-lived mutants (Δ*uga3*, Δ*uga1*, and Δ*gad1*), mutants with normal lifespans (Δ*uga2*, Δ*uga4*, and Δ*uga1* Δ*gad1*), and the wild-type strain grown in YPD and ^{1}H-NMR analysis was performed independently four times (Appendix 1). The NMR-derived metabolomic profile was visualized by orthogonal projection on latent structure-discriminant analysis (OPLS-DA) (Figure 2.4). OPLS-DA score plot indicated that all four plots from each mutant strain coalesced into clusters. It was found that the clusters separated into two large groups and that group position along axis 4 correlated with lifespan. One group contained the three long-lived mutants, Δ*uga3*, Δ*uga1*, and Δ*gad1*. The other contained the strains with normal lifespans, Δ*uga2*, Δ*uga4*, Δ*uga1* Δ*gad1*, and wild type. These results indicate that the GABA metabolic pathway mutants

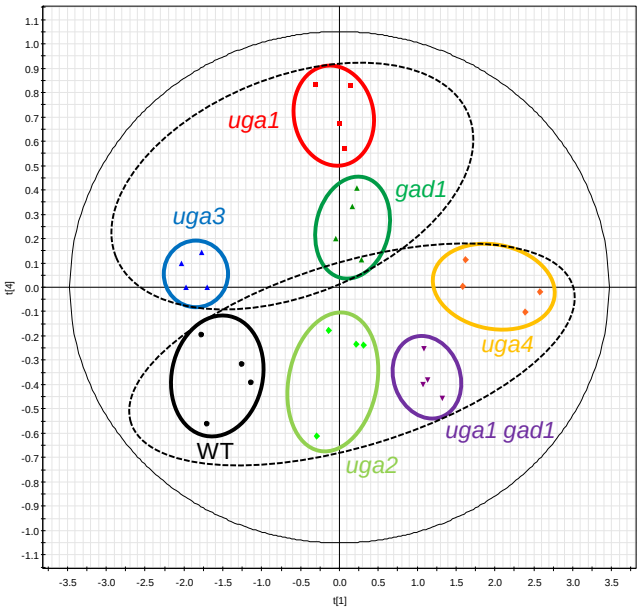

Figure 2.4 Orthogonal projection on latent structure-discriminant analysis (OPLS-DA) of ^{1}H-NMR spectra of GABA metabolic pathway mutants. OPLS-DA score plot (axis 1 vs. axis 4) is indicated. Plots from each strain coalesced into a cluster as shown by a circle. OPLS axis 4 separated the clusters into two groups, the long-lived mutants (Δ*uga3*, Δ*uga1* and Δ*gad1*) and the strains with ordinary lifespans (wild type, Δ*uga2*, Δ*uga4* and Δ*uga1* Δ*gad1*), and each group is encircled by a dashed line.

have metabolomic profiles that correlate with replicative lifespan.

2.3.5 Tricarboxylic acid cycle intermediates contribute to lifespan extension

The above data indicated that succinic acid contributed to lifespan extension and that the metabolomic profile from ^{1}H-NMR spectra correlated with longevity. To identify other specific metabolites that may affect lifespan, 57 compounds were identified and measured by GC-MS analysis (Appendix 2) and correlations between levels of individual metabolites and replicative lifespan were examined. The GABA metabolic pathway mutants were classified into the long-lived and normal-lived mutants and OPLS-DA was performed using the GC-MS data (Figure 2.5). The scatter plot (S-plot) indicates levels of three neighboring TCA cycle intermediates (succinic acid, fumaric acid, and malic acid) positively correlated with lifespan extension; however, other TCA cycle intermediates (citric acid and 2-oxoglutaric acid) did not. Interestingly, 5- aminolevulinic acid, the first compound in the porphyrin synthesis pathway that leads to heme production,

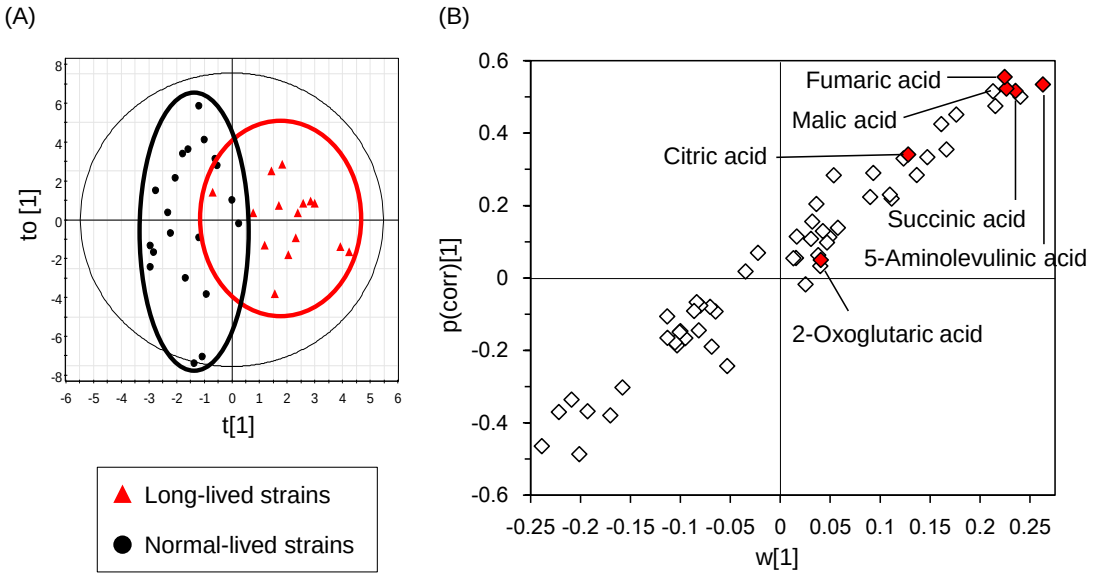

Figure 2.5 OPLS-DA of GC-MS metabolite profiles from GABA metabolic pathway mutants. (A) Score plot and (B) Scatter plot (S-plot; loading (w[1]) vs. correlation (p(corr)[1])). Plot positions of TCA cycle intermediates (succinic acid, malic acid, fumaric acid, citric acid, and 2-oxoglutaric acid) and 5-aminolevulinic acid are indicated.

also positively correlated with lifespan extension. Such a shift in carbon metabolism may cause the lifespan extension observed in Δ*uga1* and Δ*gad1* single mutants. Moreover, this hypothesis is consistent with the observation that the TCA cycle intermediates and 5-aminolevulinic acid did not increase in the Δ*uga1* Δ*gad1* double mutant, which displayed a normal lifespan.

2.3.6 Deletion of GABA-metabolizing genes does not increase respiration

Reportedly, calorie restriction extends replicative lifespan in budding yeast by increasing respiration (26). In yeast, when glucose levels are high, fermentation takes precedence over respiration. When glucose is limiting, respiration takes precedence and carbon is shunted to the mitochondrial TCA cycle, thereby increasing electron transport and respiration. The *UGA1* and *GAD1* deletions may have caused a shift in carbon metabolism toward the TCA cycle and increased respiration, as calorie restriction does.

To investigate this possibility, oxygen consumption rates of cells deleted for GABA-metabolizing genes, including *UGA3*, *UGA1*, and *GAD1*, were measured. Deletion of

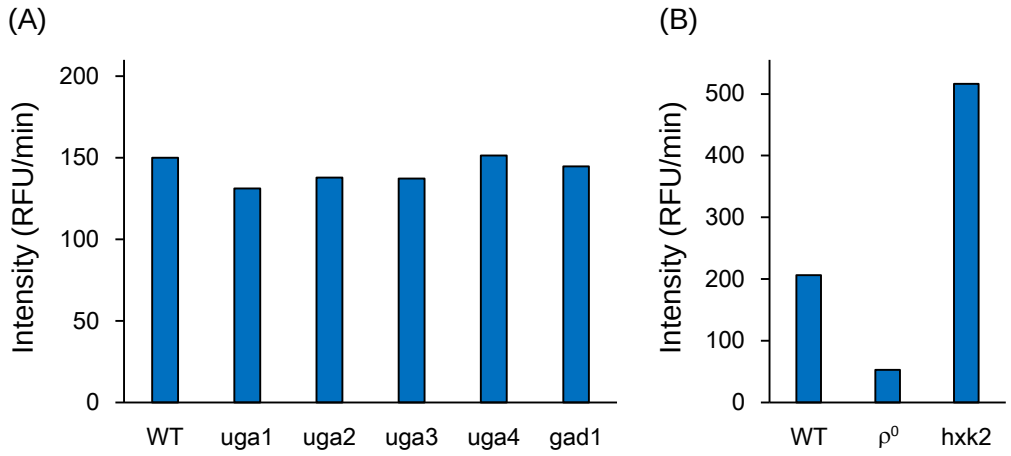

Figure 2.6 Oxygen consumption measurement. Cells deleted for GABA metabolism pathway genes (A) and carrying no mtDNA (ρ^0) or a deletion in *HXK2* (B) were grown and assayed with 2% glucose YPD medium. Results shown here represent the average of 3 experimental trials.

these GABA-metabolizing genes did not alter the oxygen consumption rate (Figure 2.6A). The oxygen consumption rate of the strain lacking mitochondrial DNA (mtDNA) (ρ^0) was decreased (Figure 2.6B). Deletion of *HXK2*, which encodes one of three hexokinases that introduce glucose into glycolysis, is expected to mimic the effect of growth in low glucose and has been shown to increase respiration rate (26). The oxygen consumption rate of the Δ*hxk2* cells was increased compared with that of wild-type cells (Figure 2.6B). These results indicated that deletion of *UGA1* and *GAD1* does not enhance mitochondrial respiration and does not cause lifespan extension through increasing TCA cycle intermediates.

2.3.7 Deletion of UGA1 extended lifespan independently of respiration

Since respiration of Δ*uga1* cells did not increase, deletion of *UGA1* is supposed to extend lifespan independently of respiratory activity. It has been reported that respiration-deficient cells experience shorter, not longer, lifespans (43). It was examined that whether deletion of *UGA1* gene in the mitochondrial respiratory dysfunction cells increase lifespan. Replicative lifespan of the Δ*uga1* strain lacking mtDNA was shorter than that of the cells with intact mtDNA (Figure 2.7). Deletion of *UGA1* in the cells lacking mtDNA

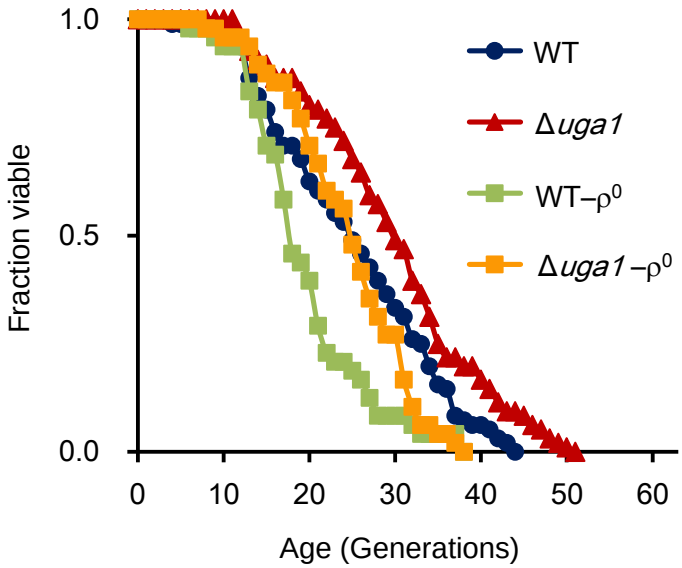

Figure 2.7 Replicative lifespans of cells without mtDNA. Lifespan of the respiration-deficient cells with or without *UGA1* deletion was determined. Average lifespans: BY4742 (wild type, WT), 25.2 generations; Δ*uga1*, 30.1; WT-ρ^0, 19.6; Δ*uga1*-ρ^0, 24.6.

increased replicative lifespan compared with *UGA1*$^+$ ρ^0 cells, indicating that deletion of *UGA1* extends replicative lifespan independently of respiration.

2.3.8 Deletion of GABA-metabolizing genes extended lifespan in SIR2-dependent manner

Lifespan extension by deletion of *UGA1* did not required respiratory function. Reportedly, one mechanism by which calorie restriction extends the replicative lifespan is activation of Sir2p, an NAD^+-dependent deacetylase, through shift in carbon metabolism toward the TCA cycle and by increased respiration (26). To examine the possibility that the *UGA1* and *GAD1* deletions cause activation of Sir2p to result in lifespan extension, the effect of deletion of the *SIR2* gene on lifespan extension by deletion of *UGA1* and *GAD1* was tested (Figure 2.8). The Δ*uga1* Δ*sir2* and Δ*gad1* Δ*sir2* strains showed short lifespan comparable with Δ*sir2* strain. These results revealed that *SIR2* is required for lifespan extension by deletion of *UGA1* and *GAD1*. This suggested that deletion of *UGA1*, probably also *GAD1*, might activate Sir2p function to extend lifespan independently of respiration.

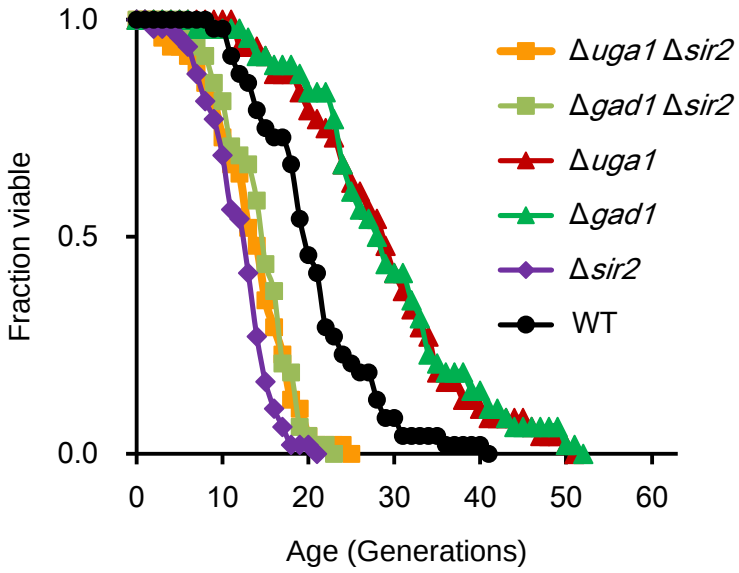

Figure 2.8 The effect of deletion of the *SIR2* gene on lifespan extension by deletion of *UGA1* and *GAD1*. Average lifespans: BY4742 (wild type, WT), 20.7 generations; Δ*uga1*, 28.9; Δ*gad1*, 29.3; Δ*sir2*, 12.1; Δ*uga1* Δ*sir2*, 13.6; Δ*gad1* Δ*sir2*, 14.6.

2.4 Discussion

Previous study showed that deletion of the *UGA3* gene—which encodes a zinc-finger transcription factor necessary for GABA-dependent induction of the *UGA1*, *UGA2*, and *UGA4* genes—extends replicative lifespan in the budding yeast. Here, it was found that deletion of *UGA1* and *GAD1*, like *UGA3*, lengthened the lifespan, indicating that the two genes in the GABA metabolism pathway were lifespan-related genes. Multivariate analysis of ^{1}H-NMR spectra for the whole-cell metabolite levels demonstrated a separation between long-lived and normal-lived strains. GC-MS analysis of identified metabolites showed that levels of TCA cycle intermediates positively correlated with lifespan extension. Respiration was not enhanced in the long-lived Δ*uga1* and Δ*gad1* strains. Lifespan extension by deletion of *UGA1* and *GAD1* required yeast sirtuin *SIR2* gene. These results suggest that reduced activity of the GABA-metabolizing enzymes extends lifespan through activation of Sir2p function independently of respiration.

Metabolome analysis in the GABA-related gene deletion mutants indicates levels of some TCA cycle intermediates (succinic acid, fumaric acid, and malic acid) positively correlated with lifespan extension, suggesting that respiration is enhanced in the long-

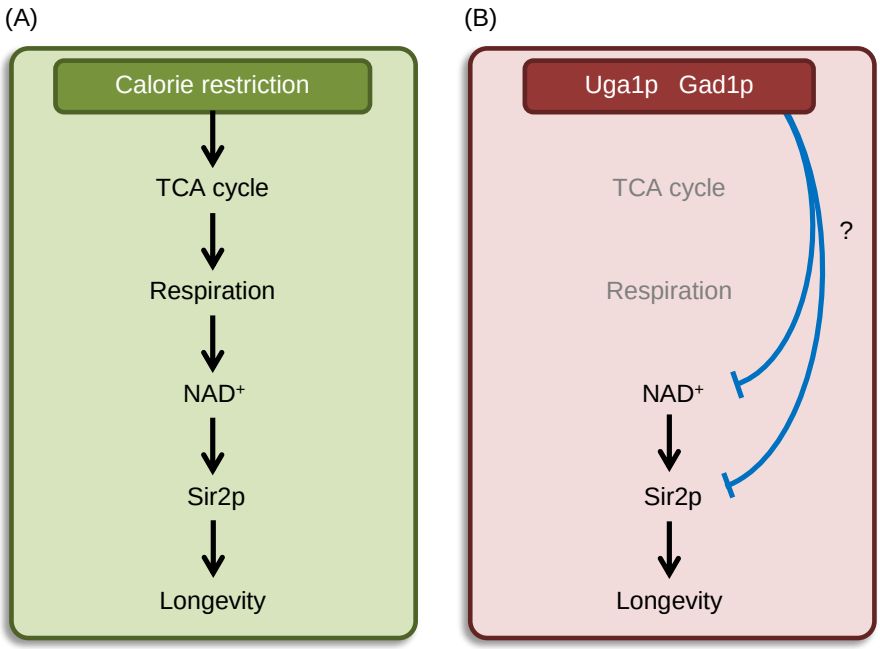

Figure 2.9 Model of lifespan regulation by GABA metabolic enzyme genes. (A) Model of lifespan extension by calorie restriction. Calorie restriction causes the shunting of carbon metabolism toward the mitochondrial TCA cycle to increase respiration. Enhanced respiration increases the cellular NAD^+, and an NAD^+-dependent deacetylase Sir2p belonging to the sirtuin family acts as a master regulator of anti-aging. (B) Model of lifespan regulation by Uga1p and Gad1p. Deletion of *UGA1* and *GAD1* results in increase of NAD^+ content or activation of Sir2p independent of respiration.

lived mutants. However, respiration was not increased in the long-lived mutants. This is different from a mechanism for lifespan extension by calorie restriction, which increases another TCA cycle intermediates (citric acid and 2-oxoglutaric acid) to enhance respiration. As the other mechanism for lifespan extension by deletion of *UGA1* and *GAD1*, Uga1p and Gad1p seem to inhibit Sir2p activity downstream of respiration (Figure 2.9). Since Sir2p activity is dependent on NAD^+ content, deletion of *UGA1* and *GAD1* would increase NAD^+ content in an unknown manner. Since malic acid content accumulates slightly in the Δ*uga1* and Δ*gad1* cells, conversion of oxaloacetic acid into malic acid is supposed to be enhanced, resulting in increase of content of NAD^+ (44).

This study is the first to demonstrate that a GABA transaminase gene regulated aging, although there are age-related changes in GABA transaminase (GABA-T) immunoreactivity in the hippocampus and dentate gyrus of the gerbil, a burrowing

mouse-like rodent (45). GABA-T protein content is elevated in gerbils in postnatal month 12, but levels decreased after that point. The GABA transaminase *UGA1* gene is upregulated between the 7th and 11th generations as described in Chapter 3 although it is unclear which generation in yeast is equivalent to the postnatal month 12 of the gerbil.

In human visual cortex, expression of the GABA synthesizing enzyme (GAD65), which is the homolog of yeast Gad1p, increases from early in life to the teen and adult years, and GAD65 levels decline in older adults (46). Similarly, the expression of yeast *GAD1* gene is increased in the early stage of cellular senescence as described below. Since transcription of the *GAD1* gene is regulated by Mtl1p, which activates general stress response during glucose starvation and oxidative stress via the transcription factor Msn2p/Msn4p (47), glutamate decarboxylase Gad1p might regulate lifespan in response to nutrient limitation and/or oxidative stress with aging.

2.5 Summary

Many of the genes involved in aging have been identified in organisms ranging from yeast to human. Previous study showed that deletion of the *UGA3* gene—which encodes a zinc-finger transcription factor necessary for GABA-dependent induction of the *UGA1*, *UGA2*, and *UGA4* genes—extends replicative lifespan in the budding yeast *Saccharomyces cerevisiae*. In this chapter, it was found that deletion of *UGA1* lengthened the lifespan, as did deletion of *UGA3*; in contrast, strains with *UGA2* or *UGA4* deletions exhibited no lifespan extension. Deletion of *GAD1* also increased lifespan. Therefore, two genes in the GABA metabolism pathway, *UGA1* and *GAD1*, were identified as aging genes. Unexpectedly, intracellular GABA levels in mutant cells (except for Δ*uga2* cells) did not differ from those in wild-type cells. Addition of GABA to culture media, which induces transcription of the *UGA* structural genes, had no effect on replicative lifespan of wild-type cells. Multivariate analysis of ^{1}H-NMR spectra for the whole-cell metabolite levels demonstrated a separation between long-lived and normal-lived strains. GC-MS analysis of identified metabolites showed that levels of TCA cycle intermediates positively correlated with lifespan extension, but respiration was not enhanced in long-lived strains. Lifespan extension due to Δ*uga1* and Δ*gad1* required yeast sirtuin gene,

SIR2. These results suggest that reduced activity of the GABA-metabolizing enzymes extends lifespan through activation of Sir2p function independently of respiration.

Chapter 3

Transcriptomic and metabolomic analyses during the early stage of replicative cellular senescence

3.1 Introduction

In the previous study described by Yoshida *et al*. (30) and in Chapter 2, metabolomic profiling indicated lifespan-correlated compounds, following identification of lifespan-related genes. These metabolomic analyses were performed by using cells in logarithmic growth phase, in which most of the cells dose not undergo cell divisions. It was surprised that metabolome data from such young cells led to the candidates of replicative lifespan-related genes. Furthermore, metabolic information of cells during the process of aging is considered to be more valuable for understanding lifespan determination.

A lot of changes in physiological phenomena during cellular senescence have been demonstrated. In mammals, senescent cells exhibit an enlarged and flattened cellular morphology and an increase in the production of reactive oxygen species (ROS) (13,15). Some of these age-related cellular phenomena are explained by time-series gene expression profiles; the senescent process is divided into four stages (early, middle, advanced, and very advanced), with specific genes being prominently expressed at each of these stages (16). Yeast cells age as they undergo division, as do cultured mammalian cells: their size increases, their shape is altered, their cell cycle slows down, and they become sterile (19). Furthermore, the nucleolus of aged yeast cells tends to be larger and/or more fragmented, and the mitochondria become dysfunctional (19,20). The rate of protein synthesis and ribosome activity decrease linearly with age (21). In mother cells during the budding process, oxidative stress, protein aggregation, and extrachromosomal rDNA circles (self-replicating circles of ribosomal DNA) accumulate and cause senescence (20).

Several transcriptome analyses comparing old cells with young cells have shown pathway changes in expression during replicative aging (25,48-50). In all cases, a shift from glycolysis toward gluconeogenesis and energy storage (glyoxylate cycle, lipid

metabolism and glycogen production) was observed in old cells. In addition, the expression of ribosome genes, and genes involved in protein synthesis, folding, and degradation, all decreased (49,50). Besides changes in metabolic gene expression, environmental stress response genes were induced in aged cells (25) although oxidative stress gene expression did not change (49). Genes involved in DNA damage repair, such as homologous recombination and nucleotide excision repair, were also induced in old cells (25,50). These transcriptome studies examined 18-20-generation-old yeast cells (i.e., approaching the end of an average lifespan) prepared by using biotin-streptavidin technology or a centrifugal elutriator (25,49,50). About half the population of cells is dead by the average generation time, so the transcriptome data from the 18th-20th-generation cells may contain information from dead cells. Alternatively, the removal of old cells with an average lifespan might result in losing information from dead cells, since yeast cells lyse at the end of their lifespan (25).

There may be few investigations focusing on the early stage of replicative aging cells. To determine what change initiates the aging process, metabolomic and transcriptomic profiles of yeast cells at the 1st, 4th, 7th, and 11th generation were generated. Distinctive changes in transcription appeared after 11 generations: decreased amino acid biosynthesis, and increased sugar and TCA cycle metabolism. These transcriptional changes were confirmed at the metabolite level. Moreover, the expression of stationary phase-induced genes was highly enhanced after 11 generations, despite the presence of adequate nutrients in the medium. These observations suggest that nutrient sensing and/or signaling begin to deteriorate in the early stage of replicative senescence.

3.2 Materials and methods

3.2.1 Strains and media

S. cerevisiae strains used in this study were BY4742 (*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*) (51) and X2180-1A (*MAT***a** *SUC2 mal mel gal2 CUP1*) (52). Yeast extract (1%), Bacto Peptone (2%), dextrose (2%) (YPD) medium was used for routine cultures.

3.2.2 Replicative lifespan determination

Replicative lifespan was assayed with minor modifications as described in Chapter 2. Yeast cells were thawed from frozen stocks and streaked onto YPD agar plates. After 2 days, a single colony was transferred to a fresh YPD agar plate, and cells were grown overnight. A sample was spread onto a new YPD agar plate containing 10 μg/mL phloxine B. Using a micromanipulator, 48 cells were arrayed on a YPD plate and allowed to undergo 1 or 2 divisions. Virgin cells were then selected and subjected to lifespan analysis. Except during manipulation, plates were sealed with Parafilm, incubated at 30°C during the day and stored at 4°C at night to avoid excessive budding. Daughter cells were removed by gentle agitation with a dissecting needle and scored every 2 h. For each of the 48-cell lines, buds from each mother cell were counted until division of living cells ceased or cells were stained with phloxine B.

3.2.3 Isolation of old yeast cells

Isolation of old cells was performed as described previously (53). Cells were grown in YPD medium to OD_{600} of 1.0. 5×10^7 cells were spun, washed with 1×PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4), and resuspended in 0.2 mL of 1×PBS. Separately, 4.0 mg of EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific, Rockford, IL, USA) was dissolved in 0.15 mL of 1×PBS at room temperature and added to the cell suspension. The mixture was shaken for 15 min by vortexing at a slow setting at room temperature. Cells were then spun and washed three times with 1 mL of 1×PBS. Biotin-labeled cells were then diluted and grown in YPD until the 4th or 7th generation, then spun down and resuspended in 20 mL of cold 1×PBS. Dynabeads Biotin Binder (Invitrogen, Carlsbad, CA, USA) were added, 4 beads per biotinylated cell. Cells and beads were rotated for 2 h at 4°C, then the suspension was placed in a test tube in a DynaMag-50 magnet (Invitrogen) at 4°C. After 20 min, the supernatant was carefully aspirated, 10 mL of cold YPD was added, and the mixture was gently agitated. The cells were again placed in the sorter for 15 min, and the process was repeated seven times. For the 11th generation cells, the isolated 7th generation cells were grown in YPD for four generations and the sorting procedure was repeated. The average bud scar count of each

sorted cell population was determined by staining an aliquot with fluorescent brightener 28 (MP Biomedicals, Illkirch, France) and counting bud scars under a fluorescence microscope.

3.2.4 Microarray analysis and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNAs were isolated from yeast cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Antisense RNA was synthesized and labeled with Cy3 for the 4th, 7th, and 11th generation and with Cy5 for the 1st generation. Each mixture of Cy3- and Cy5-labeled antisense RNA was hybridized on a 3D-Gene Yeast Oligo Chip 6K (Toray, Kanagawa, Japan). The hybridized array was scanned with a 3D-Gene Scanner 3000 (Toray). The average intensity of the background was subtracted from the detected signals, then the sample values were determined by the global normalization method (Cy3/Cy5 ratio median=1). Genes with Cy3/Cy5 normalized ratios greater than 2.0 or less than 0.5 were defined, respectively, as up- or down-regulated genes. For the 7th/4th, 11th/4th, and 11th/7th ratios, correction factors were calculated as the ratios between the global normalization values of the 1st generation of each array measured, and used for normalization of the ratios of the designated ages. The microarray data are available from the NCBI Gene Expression Omnibus (accession number GSE59797).

Extracted total RNAs were subjected to cDNA synthesis using a PrimeScript RT Master Mix (Takara, Shiga, Japan) with random primers. Quantitative PCR was carried out using a SYBR Premix Ex Taq II (Takara) on a Thermal Cycler Dice (Takara). The expression level was normalized to that of the *UBC6* gene.

3.2.5 Metabolome analysis

Samples for quantifying intracellular metabolites in yeast were prepared as described previously (30) with minor modifications. The dried yeast cells were resuspended in 1 mL of a single phase solvent mixture of methanol/water/chloroform (2.5:1:1 v/v/v) and 40 μL of a ribitol solution (8 μg/mL) as the internal standard, then homogenized and disrupted in a ball mill at 20Hz for 3 min. The extraction was carried out at 37°C for 30

min with vigorous shaking (1,200 rpm). After centrifugation at 12,000 rpm for 3 min, the supernatant (900 μL) was transferred to a new 1.5 mL microfuge tube and mixed with 400 μL of water. After centrifugation, 1 mL of the supernatant was placed in a vacuum for 4 h and dried in a freeze-dryer until dry. Derivatization of hydrophilic metabolites was carried out at 30°C for 90 min with 100 μL of methoxyamine hydrochloride in pyridine (20 mg/mL) and subsequently at 37°C for 30 min with 50 μL of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA). To analyze the compounds in the growth medium, yeast cell cultures were centrifuged (13,000 rpm, 3 min) and the supernatant was filtered through a Millex-LG filter (0.20 μm, hydrophilic, PTFE) (Millipore, Billerica, MA, USA). Supernatant (20 μL) was mixed with 1 mL of methanol/water/chloroform with ribitol (1 μg/mL) and incubated at room temperature for 10 min. The hydrophilic phase was dried as described above. Derivatization was carried out with 50 μL of methoxyamine hydrochloride and 25 μL of MSTFA. GC-MS analysis was performed as described previously (30), independently 4 times for each generation. Compounds that are not distinguishable from each other by GC-MS analysis, such as pyruvic acid and oxaloacetic acid, are described as pyruvic acid + oxaloacetic acid.

3.2.6 Multivariate analysis

GC-MS data was processed as described previously (30) and judged by principal component analysis (PCA) using the SIMCA-P+ program, version 12.0.1 (Umetrics, Malmö, Sweden).

3.3 Results

3.3.1 Experimental concepts for the early stage of replicative senescent cells

Wild-type haploid cells of *S. cerevisiae*, such as BY4742 strain, had a replicative median lifespan of 24 generations and a maximum lifespan of 43 generations (Figure 3.1). Transcriptome analysis of 18-20-generation-old wild-type cells was previously reported and showed that cellular aging is associated with a shift toward gluconeogenesis and energy storage, and a response to genome instability (25,50). The transcripts at this age, however, might include information from dead cells or exclude information from lysed

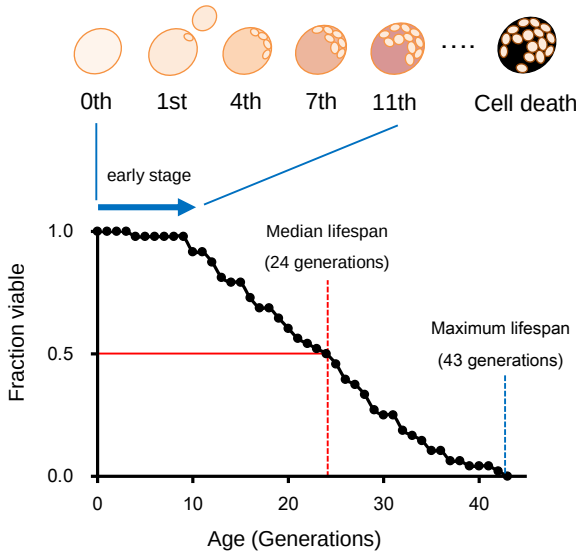

Figure 3.1 Early stage of replicative senescence in budding yeast cells. Cells of wild-type BY4742 strain have a replicative median lifespan of 24 generations, with a maximum of 43 generations. The transcriptome and metabolome of young cells (1st generation) and old cells at early stages of replicative senescence (4th, 7th, and 11th generation) were analyzed. Before 11 generations (blue arrow), almost all the cells were viable.

cells. To characterize cellular aging processes in the absence of dead cells, and to determine when cellular aging behavior begins, an earlier stage of replicative cellular senescence (approximately 10 generations), when most cells are alive (Figure 3.1) was focused. Therefore, synchronized cultures of young cells (1st generation) and older cells (4th, 7th, and 11th generation) were assessed for changes in transcription and metabolism as cells approach senescence.

Wild-type cells of designated ages were isolated by labeling the surface of mother cells with biotin and sorting the senescent cells using streptavidin-magnetic beads. Bud scars on the isolated cells of designated ages were counted to determine their generation under a fluorescence microscope after staining the cells with calcofluor (Figure 3.2). Cells from the 1st generation fraction had no bud scar (unbudded) or 1-2 bud scars, while cells from the 4th generation fraction had 3-6 bud scars. Cells from the 7th and 11th generation fractions had the highest number of bud scars, around 7 and 11, respectively. These cell fractions were used for DNA microarray and GC-MS analyses, as described below.

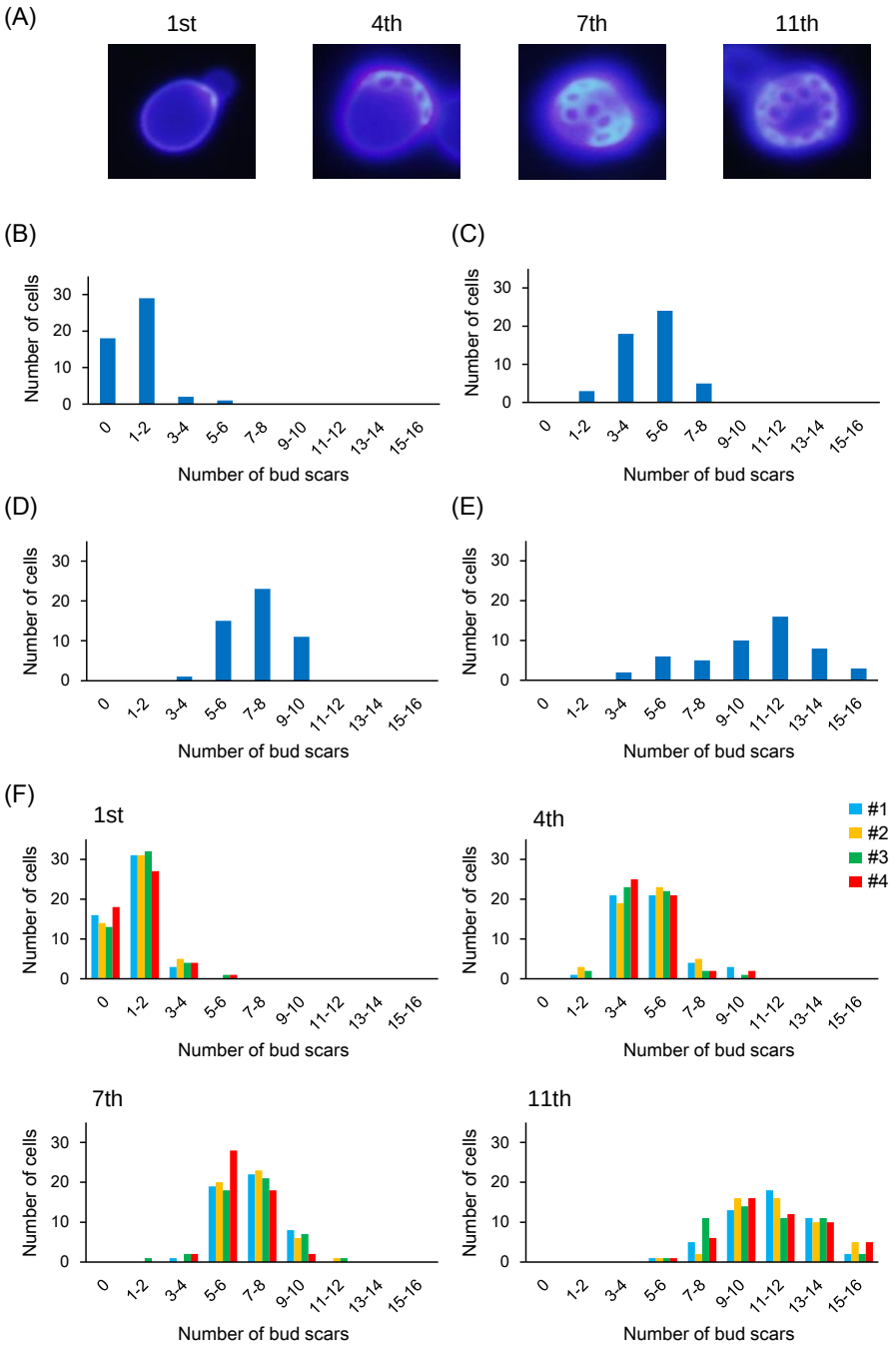

Figure 3.2 Distribution of young and old cells, isolated by generation. (A-F) Cell fractions containing the 1st, 4th, 7th and 11th generations were collected and the bud scars on cells from each fraction were stained with calcofluor. (A) Images the cells with stained bud scars from each fraction. The number of bud scars per cell was counted under a fluorescence microscope. The average number of bud scars per cell was 0.9 in the 1st fraction (B), 4.8 in the 4th fraction (C), 7.3 in the 7th fraction (D), and 10.2 in the 11th fraction (E). These cell fractions were used for transcriptomic analysis. (F) Metabolomic analysis was conducted on four independently-prepared cell fractions for each generation studied. The average number of bud scars per cell was 1.0 in the 1st fraction, 4.7 in the 4th fraction, 6.8 in the 7th fraction, and 11.1 in the 11th fraction.

3.3.2 Outline of transcriptomic changes in an age-dependent manner

Transcriptome analysis was performed by probing a DNA microarray with total RNAs extracted from the 1st, 4th, 7th, and 11th generation cells. A scatter plot of the transcriptomes of cells of designated ages versus that of the 1st generation showed moderate increases and decreases in transcripts by the 7th generation, and drastic up- and down-regulation after 11 generations (Figure 3.3A-C). The number of genes regulated in an age-dependent manner was compared between successive time points, using a threshold of a minimum two-fold change in transcript level relative to the 1st generation (Figure 3.3F and G). After 4 generations, up- and down-regulation was observed for 538 and 557 genes, respectively, for a total of about 20% of the genes in the whole genome; few further changes were observed by the 7th generation (590 and 572 genes). Genes whose expression commonly changed at the 4th and 7th generation constituted a major group among the respective generations (blue and orange bars in Figure 3.3F and G): up-regulated genes, 85% (455/538) at the 4th generation and 77% (455/590) at the 7th generation; down-regulated genes, 79% (439/557) at the 4th generation and 77% (439/572) at the 7th generation. Interestingly, after 11 generations, further up- and down-regulation was observed for 353 and 348 genes, respectively (red bars).

Large changes in the transcriptome from the 1st to the 4th generation, and small changes between the 4th and 7th generations, were observed as described above. The striking changes between the 1st and 4th generations seemed to reflect differences between non-budded (never experienced cell division) and budded cells, rather than a generation gap, since about half of the 1st generation comprised non-budded cells but all of the 4th generation cells had previously budded (Figure 3.2). To exclude transcriptional information derived from non-budded cells, transcriptomic profiles of cells of designated ages were compared with those of the 4th generation instead of the 1st generation. The scatter plot clearly showed few changes in the transcriptome of the 7th vs. 4th generation (Figure 3.3D), but large changes between the 11th and 4th generation (Figure 3.3E). These data reveal that cellular senescence can be detected as changes as early as 11 generations (i.e., at about half the median lifespan).

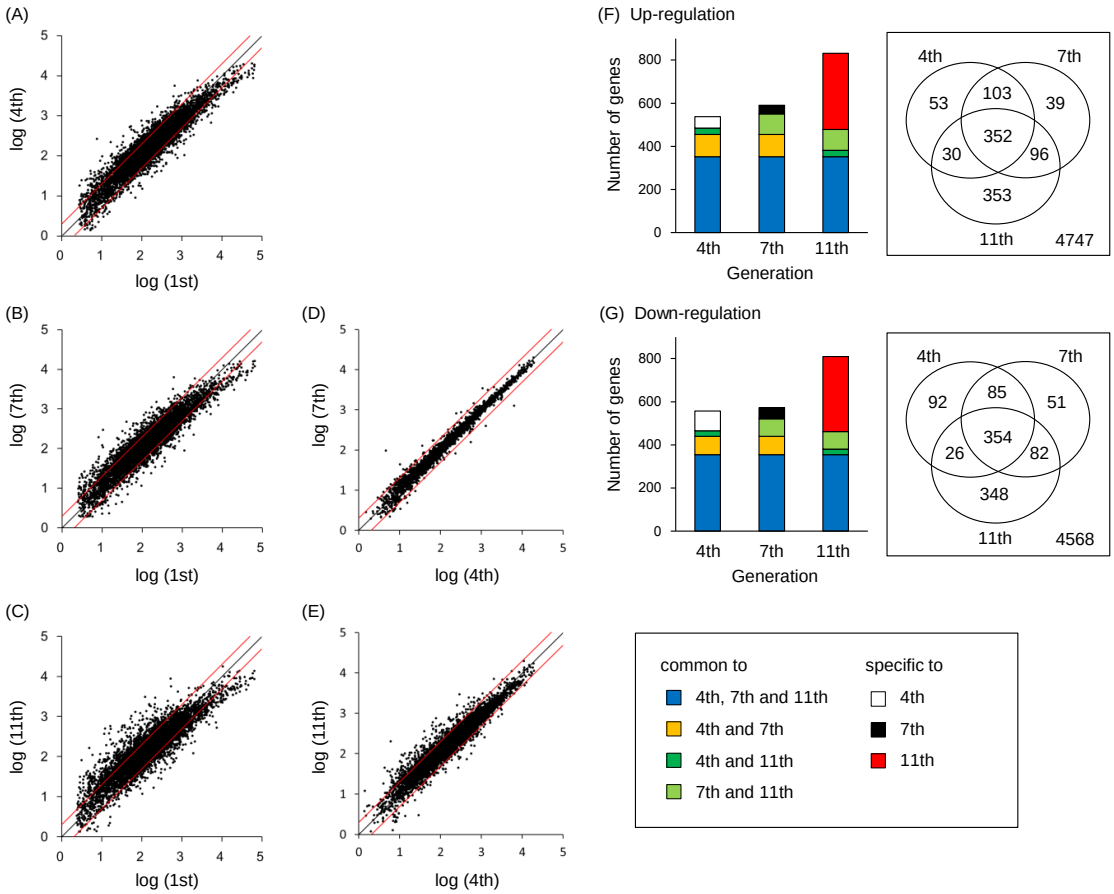

Figure 3.3 Outline of transcriptional changes occurring in an age-dependent fashion. (A-E) Scatter plots of the transcriptomes of cells; the ages being compared are indicated on each axis. Red lines indicate that the Y-coordinate/X-coordinate ratio is 0.5 or 2. (F and G) Bar graph and Venn diagram for the number of up- and down-regulated genes during the early stages of cellular aging. The number shown in the lower right corner of each Venn diagram is the number of genes whose expression was unchanged during senescence.

3.3.3 Pathways that are transcriptionally changed during the early stage of cellular senescence

To explore the functions of genes whose transcript levels change with age, the up- and down-regulated genes were sorted into categories defined by the GenMAPP database (http://www.genmapp.com/). First, genes whose transcript levels in old generations changed relative to the 1st generation were focused (Table 3.1). Few differences in pathways between the 4th and 7th generations were found; cells in the 4th and 7th generation had accumulated the same transcripts coding for aromatic amino acid (tryptophan and phenylalanine) degradative enzymes and enzymes for the biosynthesis

of sulfur amino acids (cysteine and methionine). The levels of mRNAs coding for ribosomal proteins and enzymes involved in biosynthesis of purine and pyrimidine were decreased after both the 4th and 7th generations, suggesting that the levels of ribosomal proteins and nucleic acids are higher in young cells than in old cells.

Next, pathways in which transcription changed in the 7th and 11th generation relative to the 4th generation were searched (Table 3.2). This pathway analysis clearly showed that very few pathways were changed between the 4th and 7th generations, but several biological processes and metabolic pathways were strikingly enhanced or reduced after 11 generations. Cells in the 11th generation had accumulated transcripts coding for components of the sugar metabolism and TCA cycle, consistent with previous observation of a shift from glycolysis toward gluconeogenesis in old cells (25). Unlike previous reports, this pathway analysis clearly indicates that amino acid degradation pathways were enhanced and biosynthetic pathways of branched-chain amino acid (BCAA: leucine, isoleucine, and valine) were decreased.

Previous transcriptome analyses of older cells (~20 generations) reported that environmental stress response pathways were induced in aged cells (25) although oxidative stress gene expression did not change (49). Pathway analysis in this study, however, showed no significant change in the stress response pathways of old cells, including the oxidative stress response pathway. Similarly, there were no changes in the DNA damage repair pathways, which were reported to be induced in old cells (25,50). Thus, the stress response and DNA damage repair pathways are not induced during the early stages of senescence. It was previously reported that ribosome gene expression decreased in 18-20-generation-old cells (50). However, transcriptome analysis in this study showed that the pathway for ribosomal protein expression was reduced by the 4th generation compared to unbudded cells, but was not further reduced after another 4 generations; conversely, young cells have a higher level of protein synthesis which decreases even in the early stages of aging.

Table 3.1 Pathway analysis with transcriptomic data relative to the 1st generation.

Name [a]	Number Changed [b]	Number Measured [c]	Number On MAPP [d]	Percent Changed [e]	Percent Present [f]	Z Score [g]	Permute P [h]	Adjusted P [i]
Increased								
4th								
Tryptophan degradation	5	12	16	41.7	75.0	4.17	0.00	0.22
Phenylalanine degradation	5	11	14	45.5	78.6	4.45	0.00	0.19
Glycerolipid metabolism	11	54	106	20.4	50.9	3.23	0.00	0.65
m-Cresol degradation	3	6	10	50.0	60.0	3.68	0.01	0.39
p-Cymene degradation	3	6	13	50.0	46.2	3.68	0.01	0.39
Toluene degradation	3	6	12	50.0	50.0	3.68	0.01	0.39
Fatty acid metabolism	5	17	38	29.4	44.7	3.14	0.01	0.66
Phospholipid biosynthesis	3	8	13	37.5	61.5	2.98	0.02	0.91
Sulfur amino acid biosynthesis	4	14	29	28.6	48.3	2.74	0.02	0.93
Phosphatidic acid and phospholipid biosynthesis	4	15	32	26.7	46.9	2.56	0.04	0.95
Fatty acid oxidation	3	10	16	30.0	62.5	2.47	0.04	0.97
Butanoate metabolism	6	29	69	20.7	42.0	2.41	0.04	0.97
7th								
Glycerolipid metabolism	11	54	106	20.4	50.9	3.53	0.00	0.47
Phenylalanine degradation	4	11	14	36.4	78.6	3.56	0.01	0.46
Toluene degradation	3	6	12	50.0	50.0	3.88	0.01	0.32
p-Cymene degradation	3	6	13	50.0	46.2	3.88	0.01	0.32
m-Cresol degradation	3	6	10	50.0	60.0	3.88	0.01	0.32
Tryptophan degradation	4	12	16	33.3	75.0	3.33	0.02	0.58
Sulfur amino acid biosynthesis	4	14	29	28.6	48.3	2.93	0.02	0.91
Glutamate degradation I	2	3	6	66.7	50.0	3.82	0.02	0.42
Butanoate metabolism	6	29	69	20.7	42.0	2.63	0.02	0.98
Aminophosphonate metabolism	3	11	20	27.3	55.0	2.43	0.04	0.99
Fatty acid metabolism	4	17	38	23.5	44.7	2.45	0.04	0.99
11th								
Principle pathways of carbon metabolism	21	79	98	26.6	80.6	4.36	0.00	0.06
Citrate cycle (TCA cycle)	10	30	40	33.3	75.0	3.82	0.00	0.38
Glutamate degradation I	3	3	6	100.0	50.0	4.83	0.00	0.04
Toluene degradation	4	6	12	66.7	50.0	4.27	0.00	0.07
p-Cymene degradation	4	6	13	66.7	46.2	4.27	0.00	0.07
m-Cresol degradation	4	6	10	66.7	60.0	4.27	0.00	0.07
Glycolysis / Gluconeogenesis	13	47	65	27.7	72.3	3.56	0.00	0.46
Butanoate metabolism	9	29	69	31.0	42.0	3.36	0.00	0.59
Galactose metabolism	9	30	55	30.0	54.5	3.24	0.00	0.60
Glycerolipid metabolism	13	54	106	24.1	50.9	2.98	0.00	0.74
Fatty acid metabolism	6	17	38	35.3	44.7	3.12	0.01	0.65
Fructose and mannose metabolism	9	32	79	28.1	40.5	3.01	0.01	0.74
Phospholipid biosynthesis	4	8	13	50.0	61.5	3.44	0.01	0.52
Nucleotide sugars metabolism	5	14	39	35.7	35.9	2.87	0.02	0.80
Phosphatidic acid and phospholipid biosynthesis	5	15	32	33.3	46.9	2.68	0.03	0.85
Glutamate biosynthesis	3	7	14	42.9	50.0	2.62	0.03	0.89
Glutamate metabolism	7	27	42	25.9	64.3	2.39	0.03	0.99
Oxidative branch of the pentose phosphate pathway	2	3	6	66.7	50.0	3.01	0.04	0.74

Table 3.1 Continued

Name [a]	Number Changed [b]	Number Measured [c]	Number On MAPP [d]	Percent Changed [e]	Percent Present [f]	Z Score [g]	Permute P [h]	Adjusted P [i]
Reduced								
4th								
Cytoplasmic ribosomal proteins	44	110	110	40.0	100.0	8.50	0.00	0.00
One carbon pool by folate	9	14	25	64.3	56.0	5.62	0.00	0.00
Superpathway of histidine, purine, and pyrimidine	14	38	58	36.8	65.5	4.30	0.00	0.09
Glycine degradation	3	4	11	75.0	36.4	3.62	0.01	0.38
De novo biosynthesis of purine nucleotides	7	21	37	33.3	56.8	2.70	0.02	0.87
Histidine metabolism	6	21	45	28.6	46.7	2.06	0.04	1.00
De novo biosynthesis of pyrimidine ribonucleotides	4	10	18	40.0	55.6	2.48	0.04	0.94
7th								
Cytoplasmic ribosomal proteins	55	110	110	50.0	100.0	10.37	0.00	0.00
Superpathway of histidine, purine, and pyrimidine	14	38	58	36.8	65.5	3.68	0.00	0.17
One carbon pool by folate	9	14	25	64.3	56.0	5.06	0.00	0.01
De novo biosynthesis of pyrimidine ribonucleotides	5	10	18	50.0	55.6	3.02	0.01	0.71
Glycine degradation	3	4	11	75.0	36.4	3.29	0.01	0.63
Protein modifications	5	11	15	45.5	73.3	2.75	0.01	0.78
Arginine degradation	3	4	13	75.0	30.8	3.29	0.01	0.63
Glucose fermentation	9	27	41	33.3	65.9	2.58	0.02	0.90
Glycolysis	6	16	27	37.5	59.3	2.44	0.02	0.95
De novo biosynthesis of purine nucleotides	7	21	37	33.3	56.8	2.27	0.04	0.98
Riboflavin metabolism	5	13	19	38.5	68.4	2.30	0.04	0.98
Salvage pathways of pyrimidine ribonucleotides	3	6	18	50.0	33.3	2.34	0.04	0.97
11th								
Cytoplasmic ribosomal proteins	62	110	110	56.4	100.0	8.09	0.00	0.00
One carbon pool by folate	10	14	25	71.4	56.0	4.11	0.00	0.02
Superpathway of histidine, purine, and pyrimidine	19	38	58	50.0	65.5	3.72	0.00	0.06
Glycine degradation	4	4	11	100.0	36.4	3.52	0.00	0.17
Cytoplasmic tRNA synthetases	11	19	19	57.9	100.0	3.42	0.00	0.19
Threonine biosynthesis	4	5	11	80.0	45.5	2.90	0.01	0.63
De novo biosynthesis of pyrimidine ribonucleotides	6	10	18	60.0	55.6	2.63	0.02	0.74
De novo biosynthesis of purine nucleotides	10	21	37	47.6	56.8	2.49	0.03	0.83
Protein modifications	6	11	15	54.5	73.3	2.34	0.03	0.99
Asparagine biosynthesis	3	4	9	75.0	44.4	2.36	0.04	0.99
Nitrogen metabolism	8	17	69	47.1	24.6	2.19	0.05	0.99
Lysine biosynthesis	9	20	40	45.0	50.0	2.16	0.05	1.00

[a] Gene ontology term
[b] The number of genes meeting the criterion at this node
[c] The number of genes measured at this node
[d] The number of genes associated with this node
[e] The percentage of genes meeting the criterion in this node
[f] The percentage of genes measured in this node
[g] The z score under the hypergeometric distribution
[h] The p value calculated using a non-parametric bootstrapping approach
[i] The p value using the Westfall-Young adjustment for multiple hypothesis testing

Table 3.2 Pathway analysis with transcriptomic data relative to the 4th generation.

Name [a]	Number Changed [b]	Number Measured [c]	Number On MAPP [d]	Percent Changed [e]	Percent Present [f]	Z Score [g]	Permute P [h]	Adjusted P [i]
Increased								
7th								
DNA replication	3	29	33	10.3	87.9	5.96	0.00	0.37
Pentose and glucuronate interconversions	1	9	61	11.1	14.8	3.56	0.05	0.99
11th								
Principle pathways of carbon metabolism	11	79	98	13.9	80.6	4.46	0.00	0.40
Fructose and mannose metabolism	7	32	79	21.9	40.5	5.07	0.00	0.12
Glycolysis / Gluconeogenesis	7	47	65	14.9	72.3	3.74	0.00	0.61
Butanoate metabolism	5	29	69	17.2	42.0	3.56	0.00	0.69
Galactose metabolism	5	30	55	16.7	54.5	3.46	0.01	0.69
Non-oxidative branch of the pentose pathway	3	9	18	33.3	50.0	4.39	0.01	0.41
Aminosugars metabolism	4	22	48	18.2	45.8	3.31	0.01	0.72
Nucleotide sugars metabolism	3	14	39	21.4	35.9	3.25	0.01	0.75
Valine, leucine and isoleucine degradation	3	11	38	27.3	28.9	3.85	0.01	0.61
Glycine, serine and threonine metabolism	5	42	73	11.9	57.5	2.55	0.02	0.97
m-Cresol degradation	2	6	10	33.3	60.0	3.58	0.03	0.69
p-Cymene degradation	2	6	13	33.3	46.2	3.58	0.03	0.69
Toluene degradation	2	6	12	33.3	50.0	3.58	0.03	0.69
Pentose phosphate pathway	4	26	46	15.4	56.5	2.89	0.03	0.95
Carbon fixation	3	18	29	16.7	62.1	2.67	0.03	0.96
D-Arginine and D-ornithine metabolism	2	7	15	28.6	46.7	3.24	0.03	0.92
Citrate cycle (TCA cycle)	4	30	40	13.3	75.0	2.54	0.03	0.97
Lysine degradation	4	30	67	13.3	44.8	2.54	0.04	0.97
Propanoate metabolism	2	8	48	25.0	16.7	2.96	0.04	0.94
Isoleucine degradation	2	7	8	28.6	87.5	3.24	0.04	0.92
Reduced								
11th								
Valine biosynthesis	3	5	7	60.0	71.4	7.30	0.00	0.02
Isoleucine biosynthesis	3	7	10	42.9	70.0	6.04	0.00	0.06
Valine, leucine and isoleucine biosynthesis	4	16	20	25.0	80.0	5.04	0.00	0.26
Leucine degradation	2	4	6	50.0	66.7	5.38	0.01	0.25
Pantothenate and CoA biosynthesis	3	11	30	27.3	36.7	4.61	0.01	0.40
Leucine biosynthesis	2	6	11	33.3	54.5	4.25	0.01	0.49
Isoleucine degradation	2	7	8	28.6	87.5	3.87	0.02	0.59
Ergosterol biosynthesis	3	19	31	15.8	61.3	3.18	0.03	0.89
Phenylalanine degradation	2	11	14	18.2	78.6	2.87	0.05	0.96

[a] Gene ontology term
[b] The number of genes meeting the criterion at this node
[c] The number of genes measured at this node
[d] The number of genes associated with this node
[e] The percentage of genes meeting the criterion in this node
[f] The percentage of genes measured in this node
[g] The z score under the hypergeometric distribution
[h] The p value calculated using a non-parametric bootstrapping approach
[i] The p value using the Westfall-Young adjustment for multiple hypothesis testing

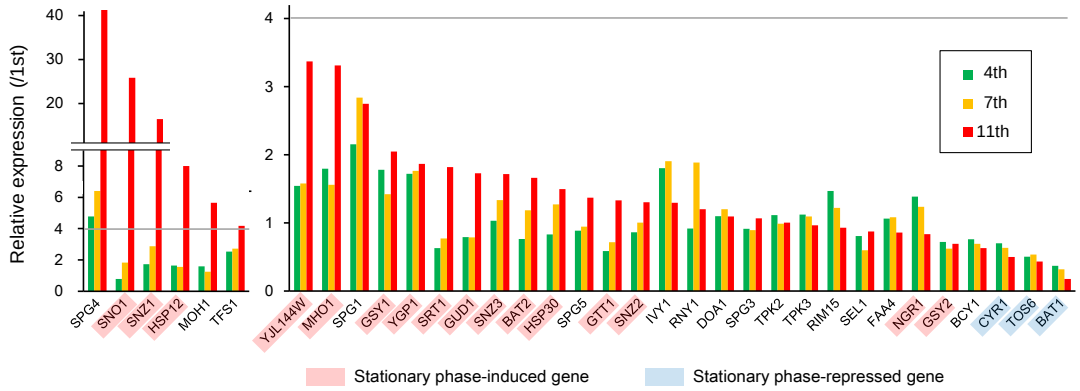

Figure 3.4 Age-dependent expression of stationary phase-related genes. Stationary phase-induced and -reduced genes are shown by red or blue shading, respectively. Expression values of designated ages relative to the 1st generation based on DNA microarray analysis are represented.

3.3.4 Genes that are highly induced by the 11th generation

In addition to analyzing age-dependent pathways, individual transcripts that were highly accumulated in 11th generation cells were focused. Among 59 genes that exhibit greater than 8-fold induction by the 11th generation relative to the 1st generation, four genes, *SPG4*, *SNO1*, *SNZ1* and *HSP12*, which are known to be induced during stationary phase (54,55) were found. The mRNA of these genes was present at more than a 5-fold higher concentration in 7th to 11th generation cells compared to 1st generation cells. The age-dependent expression was confirmed of 34 stationary phase-related genes by searching the term “stationary phase” in the *Saccharomyces* Genome Database (SGD, http://www.yeastgenome.org/) (Figure 3.4). Genes that are described as stationary-phase induced genes were mostly upregulated in aged cells, and genes that are described as stationary-phase repressed genes, such as *CYR1*, *TOS6*, and *BAT1*, were downregulated. Ten of 34 stationary phase-related genes showed more than two-fold higher expression at the 11th generation relative to the 1st generation, and nine were higher relative to the 7th generation. RT-qPCR analysis confirmed high expression of three of the top six genes and slight expression of another two genes in the 11th generation with no change in *SNO1*gene expression (Figure 3.5A). Furthermore, the age-dependent expression of 127 stationary-phase genes whose mRNA levels were reported to be reproducibly detectable in

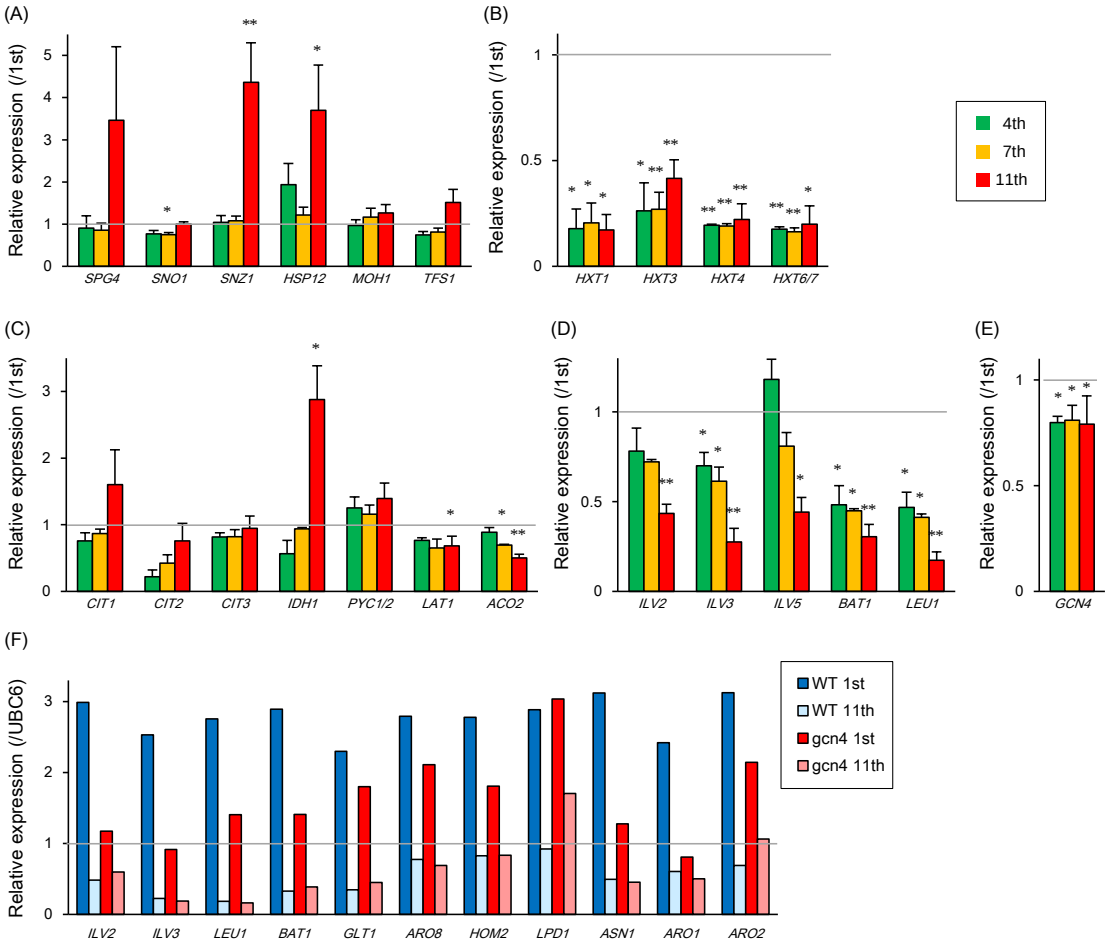

Figure 3.5 RT-qPCR confirmation of age-dependent expression changes assessed by DNA microarray analysis. The expression levels of stationary phase-related genes (A), hexose transporter genes (B), TCA cycle enzyme-encoding genes (C), BCAA biosynthetic genes (D), and *GCN4*, encoding a transcriptional activator of amino acid biosynthetic genes (E), were measured by the RT-qPCR method independently for three times. **P* < 0.05; ***P* < 0.01 vs. the 1st generation by Student's *t*-test. (F) The expression level of genes involved in amino acid biosynthesis in wild-type and Δ*gcn4* strains at the 1st and 11th generation.

stationary-phase cells was confirmed (55). About 40% of these stationary-phase genes showed more than two-fold higher expression at the 11th generation relative to the 1st generation, and about 20% were higher at the 11th generation relative to the 4th generation. These observations lead to the interesting idea that the 11th generation-induced genes overlap with stationary-phase genes and, therefore, a common transcription factor acts in both regulatory pathways.

In addition, after 11 generations, a remarkable accumulation of transcripts for 18 of the 20 genes measured belonging to the 24-gene *PAU* (seripauperin) family was observed. The gene products of the *PAU* family have unknown functions (56). *PAU* genes are highly conserved, and most of the *PAU* probes on the DNA microarray used cannot discriminate the respective *PAU* genes. However, the microarray can be used to estimate the overall expression of *PAU*. The transcription of 12 of 18 *PAU* upregulated genes clearly increased between the 7th and 11th generations. Most *PAU* gene loci are located in the subtelomeric regions of chromosomes. *PAU* genes located both in internal regions of the chromosomes as well as those in the subtelomeric regions were induced, indicating that induction of *PAU* genes is independent of a particular chromosome location, such as subtelomeric regions. Since the level of Sir2p protein, a telomere silencing factor, is known to be significantly reduced in replicatively aging yeast cells (57), some of these increases in the *PAU* expression might be due to the loss of Sir2p, at least for the subtelomeric set of genes.

3.3.5 Outline of metabolic changes occurring in an age-dependent manner

The findings from the above transcriptome analysis strongly suggested that metabolic changes begin at an early stage of replicative senescence. To confirm this, 37 low-molecular-weight intracellular compounds (including amino acids, organic acids, and sugars) were extracted from cells of designated ages and identified and quantified using GC-MS (Appendix 3). Principal component analysis (PCA) was performed to visualize significant effects with multivariate data of the profiles expressed as relative levels of the metabolites (Figure 3.6). A scores plot where the data points were projected onto a plane defined by the first principal component (PC1) and the second principal component (PC2) showed clustering of the data points from each successive generation. The generation clusters were separated from each other in both PC directions: PC1 and PC2 accounted for 58% and 13% of the total variance, respectively (Figure 3.6A). Interestingly, the variance along PC2 appeared to be correlated with the generations, with higher scores for older cells than younger cells. This indicates metabolic shifts that correlate with aging.

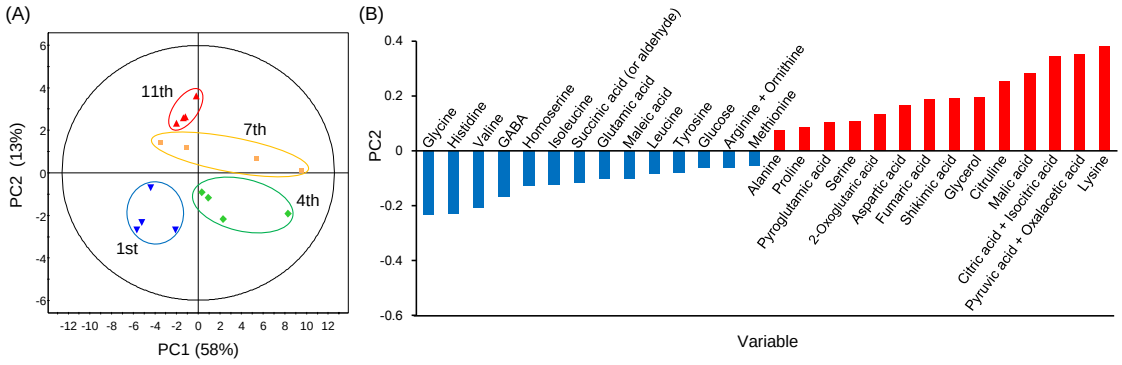

Figure 3.6 Principal component analysis (PCA) of metabolites in cells at the early stages of senescence. (A) A scores plot from PCA. Each point represents an individual batch from the designated age. (B) A loadings plot along PC2 contributing to separation of each generation. The lower and upper loading values of the metabolites were below -0.05 and above 0.05, respectively.

To identify the metabolites associated with aging, the metabolites that contributed to the separation of generations along PC2 in the PCA were searched. The relevant loading plots of PC2 represented the relative degree of correlation between the levels of each metabolite and aging (Figure 3.6B). High levels of pyruvic acid and TCA cycle intermediates (oxaloacetic acid, citric acid and isocitric acid, malic acid, fumaric acid, and 2-oxoglutamic acid) positively correlated with older generations (loading on PC2 > 0.05), while low levels of about half of the amino acids (glycine, histidine, valine, GABA, homoserine, isoleucine, glutamic acid, leucine, tyrosine, arginine, ornithine, and methionine) negatively correlated with aging (loading on PC2 < -0.05).

The metabolic profiles of the 37 compounds measured in this study were mapped on the TCA cycle and amino acid biosynthetic pathways (Figure 3.7). The concentrations of most of the metabolites varied during the early stages of senescence, as expected from PCA. Some TCA cycle intermediates increased and about half of the amino acids decreased in concentration. Only lysine accumulated in an age-dependent fashion. Some organic acids (lauric acid, maleic acid, 2-oxoglutaric acid) and amino acids (alanine, arginine, and ornithine) did not change significantly ($p > 0.05$), whereas aromatic amino acids (phenylalanine, tyrosine, and tryptophan), which are biosynthesized in the shikimate pathway, decreased slightly by the 11th generation. Both the metabolomic and

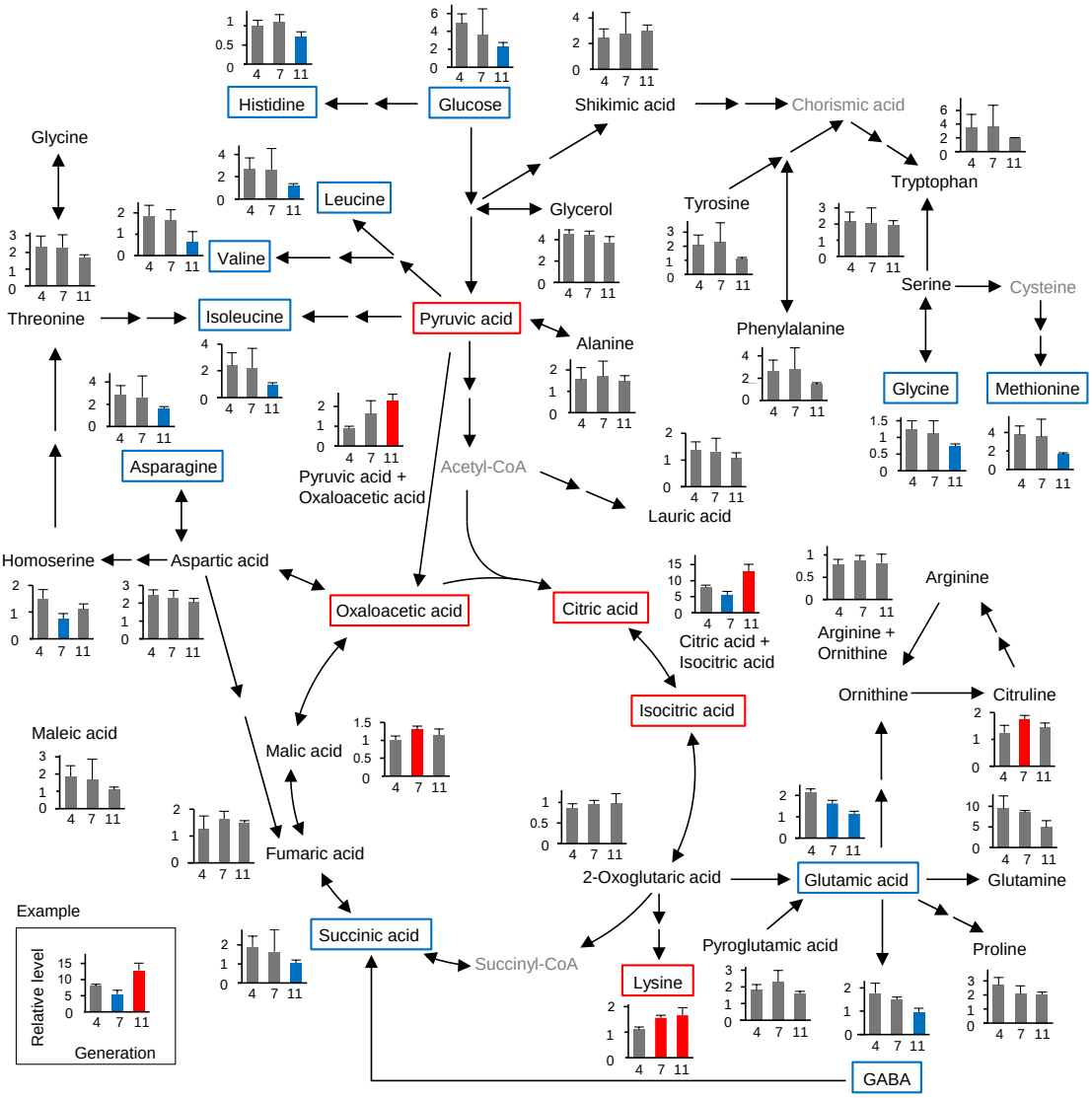

Figure 3.7 A metabolic map of central carbon and amino acid metabolism during cellular aging. Bar graphs indicate the amount of the metabolite relative to the 1st generation. For each metabolite, blue and red bars represent a significant decrease and increase, respectively, relative to the level in the 4th generation.

transcriptomic profiles revealed increased metabolite levels between generation 1 and generation 4, but exhibited little further change by generation 7. Therefore, the significance of each metabolite was determined in 7th and 11th generation cells relative to 4th generation cells; red bars and blue bars in Figure 3.7 indicate a significant increase and decrease, respectively ($p < 0.05$). After 11 generations, significant changes were observed: enhanced TCA cycle biosynthesis and decreased amino acids biosynthesis,

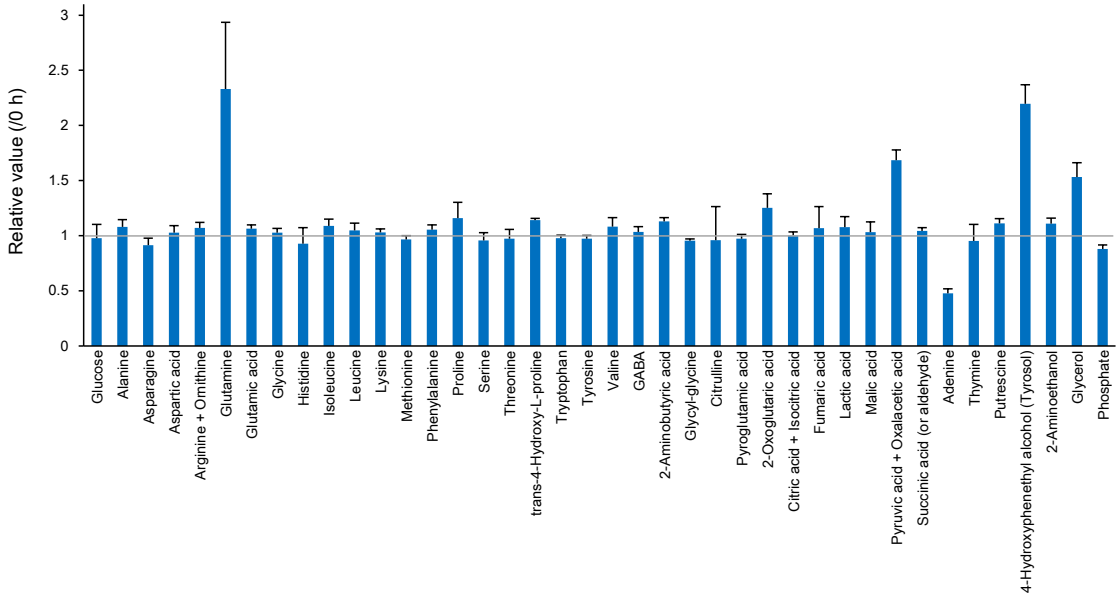

Figure 3.8 Quantification of compounds in the medium after cultivation of yeast cells. The compounds in fresh YPD medium (0 h, OD_{600}=0.01) and in cell-free culture medium after 11 h cultivation of X2180-1A yeast strain cells (OD_{600}=1.3, corresponding to 7 generations) were identified and quantified by GC-MS analysis for three independent experiments. Relative values of the average amount of each compound with standard deviations are indicated.

especially BCAA (details described below).

Additionally, intracellular glucose was reduced after 11 generations (Figure 3.7). No reduction in the concentration of glucose in the cell-free culture media was expected after 11 generations because the 11th generation cell culture was prepared by exchanging the 7th generation culture medium with fresh medium and culturing for a further 4 generations. As expected, GC-MS analysis of the cell-free yeast cell culture medium showed no difference in glucose content before and after cultivation (Figure 3.8). Accordingly, the age-dependent decrease in intracellular glucose suggested that glucose uptake decreased in senescent yeast cells. It was found in DNA microarray analysis and confirmed by RT-qPCR analysis that genes encoding glucose transporters which belongs to the major facilitator superfamily that are typically highly expressed, such as *HXT1*, *HXT3*, *HXT4*, and *HXT7* (58), were notably downregulated between the 1st and 4th generations, and that low-level transcription was sustained during the early stage of senescence (Figure 3.5B). Like *PAU* genes, *HXT* genes are highly conserved, and it is

likely that the DNA microarray for *HXT* overestimated gene expression. This indicates that a decrease in the ability to uptake glucose results in lower intracellular glucose despite there being sufficient glucose in the medium.

3.3.6 Integrating metabolic and transcriptional profiling in the TCA cycle and BCAA biosynthetic pathway

The observation that transcripts coding for components of the TCA cycle, and that TCA cycle metabolites accumulated in 11th generation cells, led to the examination of the relation between the level of TCA cycle intermediates and the transcript level of the gene coding the enzyme that catalyzes the corresponding reaction in the TCA cycle. Pyruvic acid and three neighboring TCA cycle intermediates (oxaloacetic acid, citric acid, and isocitric acid) significantly increased after 11 generations. Citrate synthase catalyzes the condensation of acetyl coenzyme A and oxaloacetic acid to form citric acid and is the rate-limiting enzyme in the TCA cycle (59,60). Interestingly, the transcript levels of the *CIT1*, *CIT2*, and *CIT3* genes, which encode citrate synthase, are increased in senescent cells according to DNA microarray analysis (Figure 3.9A) and the *CIT1* upregulation was confirmed by RT-qPCR analysis (Figure 3.5C). This may explain the increase in intracellular citric acid and isocitric acid by the 11th generation. The high level of oxaloacetic acid by the 11th generation might be caused by upregulation of the *PYC1* gene, which encodes pyruvate carboxylase that converts pyruvic acid to oxaloacetic acid (61), although the *PYC2* gene, a paralog of *PYC1*, was downregulated. Downregulation of the *LAT1* gene, which encodes a component of the pyruvate dehydrogenase complex that catalyzes the oxidative decarboxylation of pyruvic acid to acetyl-CoA (62), might contribute to the accumulation of pyruvic acid after 11 generations. These data indicate that the accumulation of several TCA cycle intermediates is under the transcriptional control of the corresponding metabolic genes. The accumulation of TCA cycle intermediates suggests that TCA cycle biosynthesis might be enhanced by aging, resulting in a higher respiration rate in aging cells. However, a gross upregulation of oxidative phosphorylation genes were not found, although several *COX* (cytochrome c oxidase) genes were upregulated. This indicates that yeast cells do not shift toward respiration with

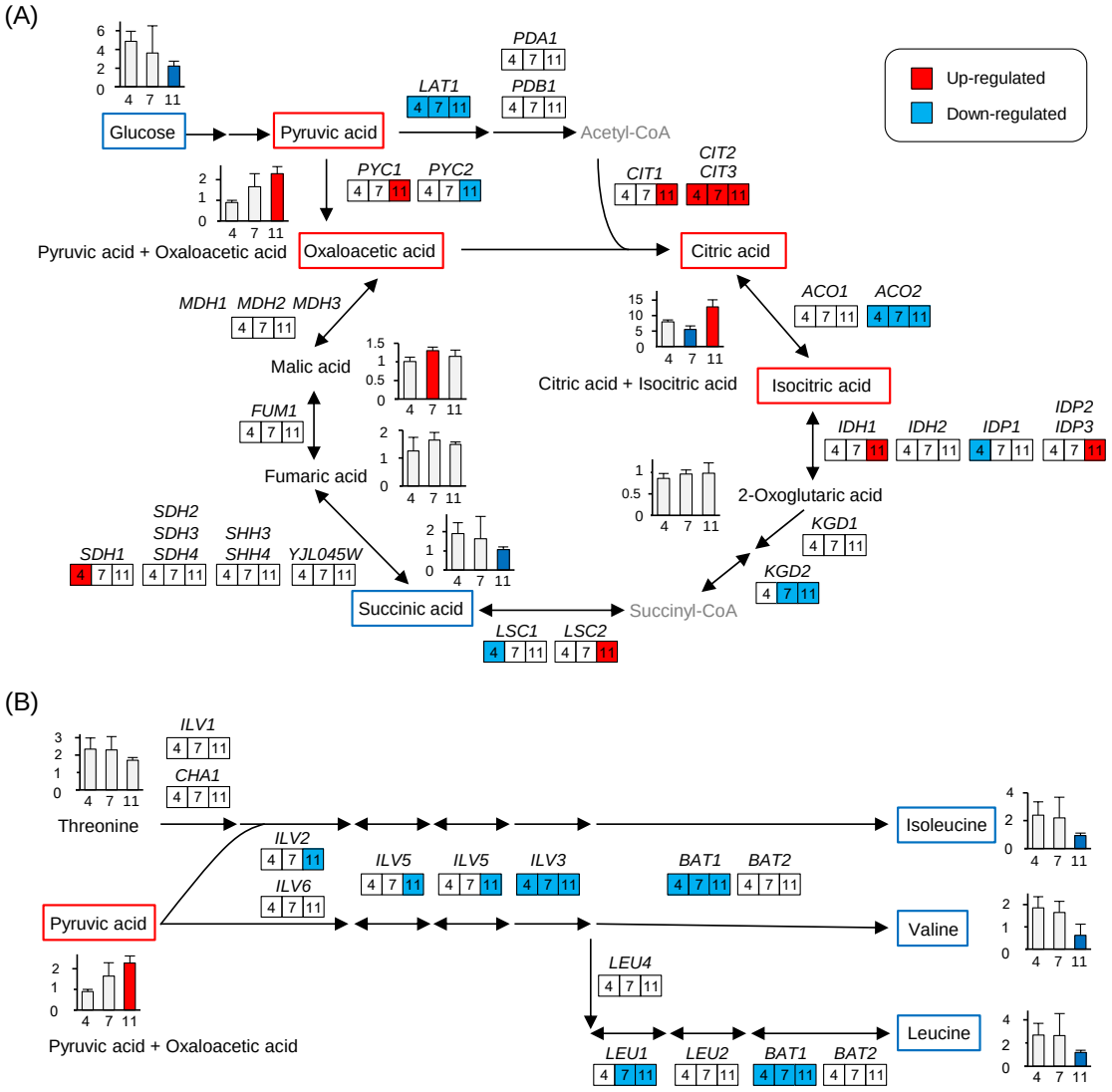

Figure 3.9 Metabolic and transcriptional profiles for components of the TCA cycle (A) and BCAA biosynthetic pathway (B). Bar graphs show the amount of the metabolite relative to the 1st generation. A significant increase or decrease relative to the level in the 4th generation ($p < 0.05$) is represented by a red or blue bar, respectively. Enhanced and reduced expression of the designated enzyme gene is shown as red and blue boxes labeled with the generation number, respectively.

aging.

The role of transcriptional regulation in the decrease of many amino acids, especially BCAA was also confirmed (Figure 3.5D). Analysis of the products of the BCAA biosynthetic pathway showed significantly decreased levels of isoleucine, valine, and leucine at the 11th generation, although the concentration of pyruvic acid, the initial

metabolite of this pathway, increased gradually with aging (Figure 3.9B). Transcript levels of most of the BCAA biosynthetic pathway genes decreased, as suggested by the above pathway analysis with transcriptomics. This clearly indicates that the low level of BCAA at the 11th generation is caused by downregulation of the BCAA biosynthetic pathway genes. A decrease in glutamic acid and GABA was observed after 11 generations (Figure 3.7). This decrease can be explained by reduced mRNA levels of the *GLT1* gene, which encodes glutamate synthase, the enzyme that catalyzes the synthesis of glutamic acid from glutamine and 2-oxoglutaric acid (63), and by increased mRNA levels of the *GAD1*, *UGA1*, *UGA2* genes, which encode components of the glutamic acid degradation pathway (34,41). Alternatively, there could also be post-transcriptional changes in enzyme stability or activity which led to the change of amino acids as well as TCA cycle intermediates observed in metabolomics analysis.

A decrease in amino acid concentrations can be explained by reduced transcript levels of the corresponding amino acid biosynthetic genes, rather than by downregulation of the amino acid transporter genes (whose transcription did not change with aging). The expression of the *GCN4* gene that encodes a transcriptional activator of general amino acid biosynthetic genes was examined. The expression of *GCN4* was comparable between designated ages of wild-type cells (Figure 3.5E). Next, in wild-type and *GCN4*-deletion mutant strains, senescence-associated expression was compared (Figure 3.5F). Deletion of *GCN4* decreased the transcription of amino acid biosynthetic genes at the 11th generation compared to the 1st generation. Thus, *GCN4*-independent reduction of amino acid biosynthetic gene transcription during replicative senescence was observed, suggesting that other transcription factors regulate age-dependent expression of amino acid biosynthetic genes.

Since intracellular amino acids decreased significantly in 11-generation-old cells, the nutrients in the culture medium were analyzed. Most nutrients were not depleted even after 11 generations, as described above (Figure 3.8), suggesting that yeast cells exhibit decreased nutrient sensing and/or signaling by the 11th generation.

3.4 Discussion

The transcriptional and metabolic profiling of yeast cells at the early stages of senescence (4th, 7th and 11th generation) was performed. Previous transcriptomic studies had analyzed older cells, close to the median replicative lifespan (18th-20th generation) (25,48-50). The transcriptional profiles showed remarkable up- and down-regulation of gene expression after 11 generations. The 11th generation cells had increased levels of pyruvic acid and TCA cycle intermediates and decreased levels of amino acids, especially BCAA. An apparent relation was observed between metabolites and transcripts of the corresponding metabolic genes. Furthermore, high expression of *PAU* family and stationary phase-induced genes was found after 11 generations, even though the yeast cells were cultivated under aerobic conditions and the growth medium contained sufficient nutrients. These changes are presumably early indications of replicative senescence.

The transcriptomic and metabolomic analyses in this study clearly indicate that replicative senescence of yeast cells begins around the 11th generation, which is about half the average replicative lifespan (Figure 3.10). Since yeast cells begin to die by the 10th generation as shown in Figure 3.1, this generation appears to be the start point for cellular senescent behavior. It was reported that 20-generation-old cells exhibited enhanced gluconeogenesis and energy storage (25), therefore, 11-generation-old cells might be just starting to switch sugar metabolisms, consistent with previous observations during the early stage of aging (48,49). Transcriptomic changes at the early stages of senescence imply the existence of transcription factors that regulate gene expression at this stage. High expression of stationary phase-induced genes after 11 generations indicates that transcription factors induce gene expression during stationary phase and regulate the transcription of senescence-induced genes. For example, Msn2p/Msn4p and Gis1p in the Rim15p protein kinase pathway, and Adr1p, Cat8p, and Mig1p in the Snf1p protein kinase pathway, are thought to be transcription factors that regulate stationary phase-induced genes (64,65). Transcription of the *MSN2*, *MSN4*, *GIS1*, and *CAT8* genes was not regulated during the early stage of senescence, but the *ADR1* activator gene was upregulated four-fold and *MIG1* repressor gene was downregulated two-fold at the 11th

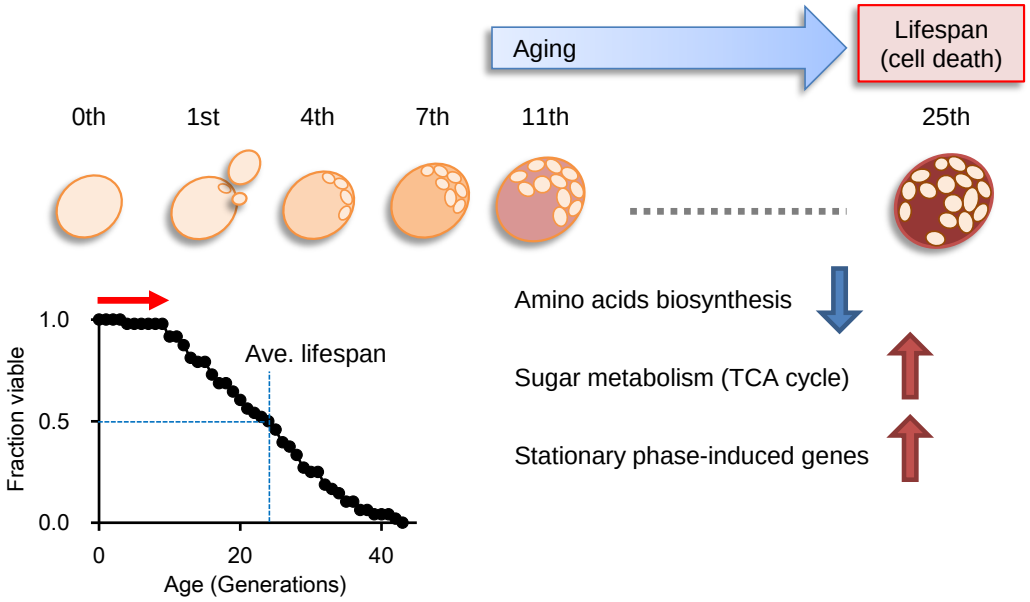

Figure 3.10 Summary of the transcriptional and metabolic profiling of yeast cells at the early stages of senescence. At the 11th generation, amino acid biosynthesis decreased, and sugar and TCA cycle metabolism increased, and the expression of stationary phase-induced genes was highly enhanced.

generation relative to the 1st generation. Thus, Adr1p and Mig1p might be required for the induction of age-associated genes, although the possible existence of an unidentified transcription factor for senescence-specific induction is not excluded. Using transcriptome data in this study, the age-associated transcription of 189 genes encoding DNA-binding transcription factor, which are searched in SGD was analyzed. Thirty-eight of these transcription factor genes were upregulated more than two-fold in 11th generation cells and twenty-six were downregulated; these genes might include senescence-associated transcription factor genes and needs to be investigated in future studies.

What deterioration begins during the early stage of senescence in yeast? It was found that 11th generation cells highly express stationary phase genes that are induced by nutrient deprivation (64). One possible trigger of cellular senescence is that a nutrient sensing and/or signaling pathway degrades with aging. The transcriptional regulation of *ADR1* and *MIG1* is unknown; however, the observed changes in their transcription may suggest that the Snf1p signaling pathway deteriorates during the early stage of senescence, even in medium containing sufficient nutrients, resulting in induction of stationary-phase

genes. *PAU* genes are induced by anaerobiosis through an unknown transcription factor(s) (66). Upregulation of *PAU* genes causes degeneration of the oxygen-responding pathway. Tup1p corepressor is required for transcriptional repression of *PAU* by oxygen. In contrast, Rox1p, a DNA-binding repressor involved in oxygen-dependent repression of other target genes such as *ANB1*, is not required (67). *TUP1* and *CYC8*, two subunits of Tup1p-Cyc8p corepressor complex, were slightly downregulated in microarray experiment (70% at the 11th generation relative to the 1st generation). This may be one reason why *PAU* genes are upregulated in senescent cells.

Another possible mechanism triggering cellular senescence is mitochondrial dysfunction. Expression of the *CIT* genes, which are controlled by retrograde transcription factors Rtg1p and Rtg3p, drastically increased after 11 generations. Retrograde regulation is triggered by mitochondrial dysfunction and the signal is transduced from mitochondria to the nucleus (68). Yeast petite mutants lacking mitochondrial DNA upregulate TCA cycle enzyme-encoding genes, including *CIT* genes (69). Mitochondrial fragmentation begins to occur in cells after 4 generations and increases as cells age, and mitochondria are severely fragmented with no tubular structure at the 11th generation (70). These observations are consistent with the observations of aging cells at the 11th generation. It is proposed that age-associated mitochondrial dysfunction is one cause of transcriptional and metabolomic changes at the early stages of replicative senescence.

3.5 Summary

Age-related damage accumulates and a variety of biological activities and functions deteriorate in senescent cells. However, little is known about when cellular aging behaviors begin and what cellular aging processes change. Previous research demonstrated age-related mRNA changes in budding yeast by the 18th-20th generation, which is the average replicative lifespan of yeast (i.e., about half the population is dead by this time point). Here, transcriptional and metabolic profiling was performed for yeast at early stages of senescence (4th, 7th and 11th generation), that is, for populations in which most cells are still alive. Transcriptional profiles showed up- and down-regulation

for about 20% of the genes profiled after the first 4 generations, few further changes by the 7th generation, and an additional 12% of the genes were up- and down-regulated after 11 generations. Pathway analysis revealed that these 11th generation cells had accumulated transcripts coding for enzymes involved in sugar metabolism, the TCA cycle and amino acid degradation, and showed decreased levels of mRNAs coding for enzymes involved in amino acid biosynthetic pathways. These observations were consistent with the metabolomic profiles of aging cells: an accumulation of pyruvic acid and TCA cycle intermediates, and depletion of most amino acids, especially branched-chain amino acids. Stationary phase-induced genes were highly expressed after 11 generations even though the growth medium contained adequate levels of nutrients, indicating deterioration of the nutrient sensing and/or signaling pathways by the 11th generation. These changes are presumably early indications of replicative senescence.

Chapter 4

Replicative lifespan regulation by vitamin B6

4.1 Introduction

As an environmental factor to regulate lifespan, calorie restriction is well known to extend lifespan in a variety of species from yeast to mammals (8,71). In yeast, calorie restriction can be modeled by reducing glucose content of the medium. It is also reported that the contents of amino acids on the medium have the influence on yeast lifespan (72). In addition to the effects of the source of carbon and nitrogen on lifespan, NAD^+ increases histone deacetylase activity of yeast sirtuin family protein Sir2p, which is well known as a replicative lifespan regulator (31). However, effects of other physiologically active compounds, such as vitamins, on lifespan are little known. For example, there is no consistent beneficial effect of vitamin E on lifespan in model organisms which is consistent with reports in human intervention studies (73-80).

Transcriptional profiling of aging cells as described in Chapter 3 showed that some of the stationary phase-induced genes, such as *SNZ1* and *SNO1*, were highly expressed at the 11th generation (81). Transcriptional level of the *SNZ1* gene, which encodes pyridoxal 5'-phosphate (PLP) synthase (82), and the *SNO1* gene, which encodes glutamine amidotransferase (83), greatly increased in old cells. Since Snz1p and Sno1p form a complex to synthesize PLP from glyceraldehyde 3-phosphate and ribulose 5-phosphate (Figure 4.1) (82), it is hypothesized that PLP, the major active form of vitamin B6, is involved in replicative lifespan. Vitamin B6 contains pyridoxine (PN), pyridoxal, and pyridoxamine, and PLP serves as a cofactor in many enzyme reactions in amino acid, glucose, and lipid metabolism (84). In addition, Tpn1p is known to transport extracellular vitamin B6 (85). The function of the vitamin B6 biosynthesis and uptake genes in regulation of lifespan is unknown.

In this Chapter 4, the role of vitamin B6 on replicative lifespan regulation was investigated. *SNZ1* and *TPN1* was involved in replicative lifespan regulation. Supplementation of excess pyridoxine restored replicative lifespan of Δ*snz1* and Δ*tpn1*

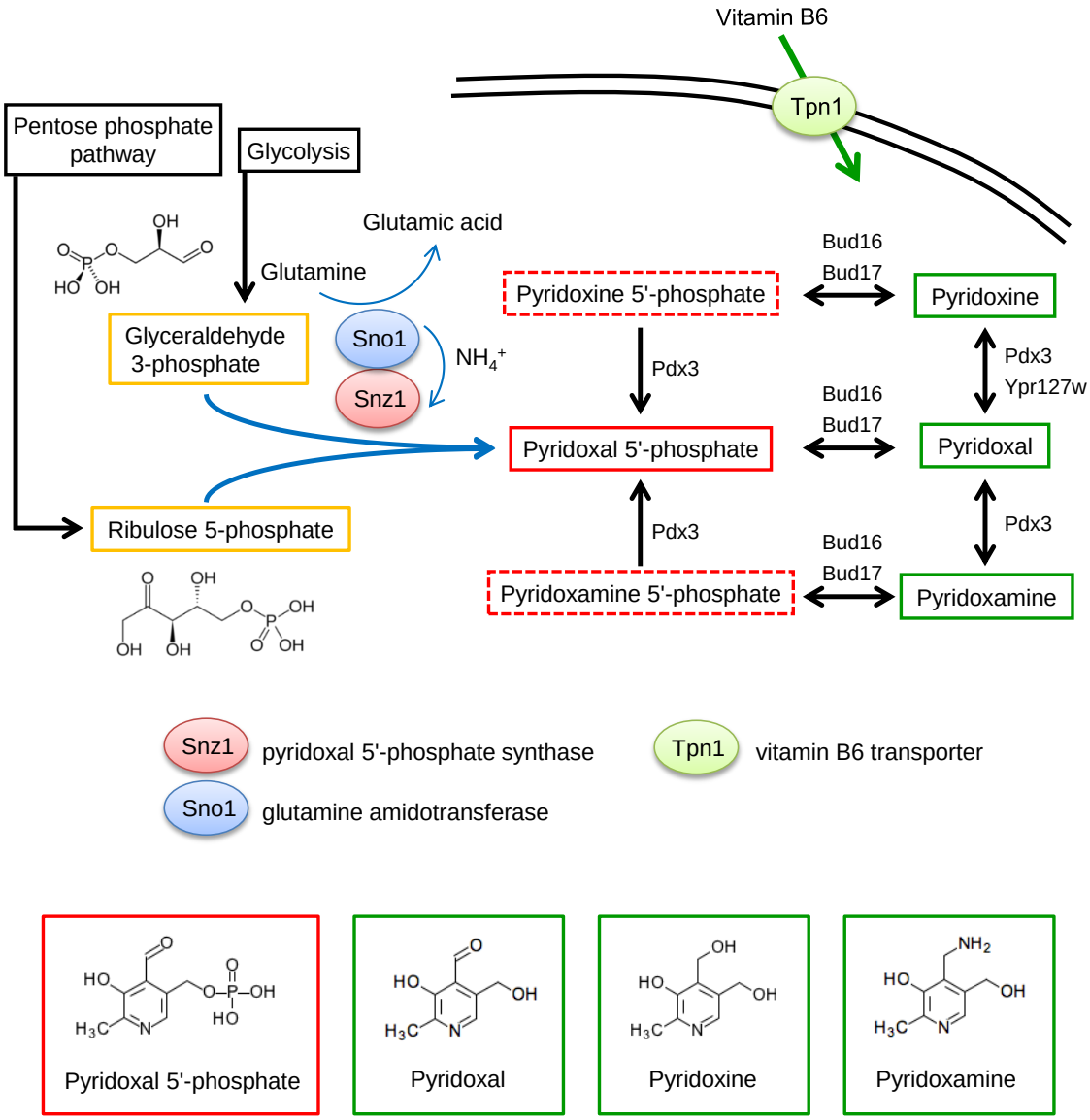

Figure 4.1 Biosynthesis and uptake of vitamin B6. Vitamin B6 contains pyridoxine, pyridoxal and pyridoxamine. Pyridoxal 5'-phosphate (PLP), the major active form of vitamin B6, serves as a cofactor in many enzyme reactions. A complex of Snz1p and Sno1p synthesizes PLP from glyceraldehyde 3-phosphate and ribulose 5-phosphate. Tpn1p transports extracellular vitamin B6.

cells. Depletion of extracellular vitamin B6 had no effect of wild-type cells on replicative lifespan. These observations suggest that intracellular vitamin B6 is important for longevity. Additionally, a transcription factor Adr1p was required for age-dependent induction of *SNZ1* transcription. The significance of Adr1p for discovering a candidate trigger of cellular senesce will be discussed.

Table 4.1 Yeast strains used in this study.

Strain	Genotype
BY4742	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*
BY2-spg4	*MATα spg4Δ::kanMX ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*
BY2-snz1	*MATα snz1Δ::kanMX ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*
BY2-sno1	*MATα sno1Δ::kanMX ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*
BY2-tpn1	*MATα tpn1Δ::kanMX ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*
BY2-snz2-snz3	*MATα snz2Δ::CgHIS3 snz3Δ::kanMX ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*
BY2-sno2-sno3	*MATα sno2Δ::kanMX sno3Δ::CgHIS3 ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*
BY2-GpSNZ1	*MATα GAL1-SNZ1::CgLEU2 ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*
BY2-PpSNZ1	*MATα PHO4-SNZ1::CgLEU2 ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*

4.2 Materials and methods

4.2.1 Strains and media

All *S. cerevisiae* strains used in this study were derived from BY4742 (Table 4.1). Deletion strains were obtained from the MATα ORF deletion collection (Open Biosystems, USA). YPD medium (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% dextrose) was used for routine cultures. Synthetic defined minimal medium contained 20 g glucose, 2 g asparagine, 1mL of vitamin mix stock solution [nicotinic acid, thiamine, pantothenic acid (200 mg each), inositol (10 g), biotin (20 mg)], the amino acid solution and 250 mL of high-Pi stock solution [6 g KH_2PO_4, 2 g $MgSO_4.7H_2O$, 1.32 g $CaCl_2.2H_2O$, 0.4 mg KI] and trace elements [B, Cu, Mn, Mo (0.04 mg each), Fe (0.2 mg), Zn (0.28 mg)] per liter. Pyridoxine was added to give the desired concentrations. *Saccharomyces carlsbergensis* strain BY4609 (ATCC9080, IFO0565), auxotrophic for vitamin B6, was used for vitamin B_6 microbiological assay. YM medium (0.3% Bacto Yeast Extract, 0.3% Malt Extract, 0.5% Bacto Peptone, 1% dextrose) was used for cultivation of *S. carlsbergensis*.

4.2.2 Replicative lifespan determination

Replicative lifespan was assayed as described in Chapter 3. Synthetic defined minimal agar plates containing pyridoxine (2 μM) without phloxine B were used as indicated in the text.

4.2.3 Evaluation of pyridoxine requirement

To evaluate pyridoxine requirement, yeast strains were grown in YPD medium to OD_{600} of 1.0. The growth medium was diluted to a final OD_{600} of 1.0 and then diluted by 1/5 for a series of six dilutions. Then, each of these dilutions was plated using a 48-pin replicator onto YPD medium, synthetic defined minimal medium supplemented with pyridoxine, when indicated (see Results), for 2 days at 30°C.

4.2.4 Isolation of old yeast cells

Isolation of old cells was performed as described in Chapter 3.

4.2.5 Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA isolation and RT-qPCR experiment were performed as described in Chapter 3. The expression levels of senescent cells was normalized to that of the *UBC6* gene and those of stationary-phase cells was normalized to that of *RDN18* gene.

4.2.6 Determination *of vitamin B6 content*

A microbiological assay with a yeast *Saccharomyces carlsbergensis* strain BY4609 (ATCC9080, IFO0565) that is auxotrophic for vitamin B6 was used to determine total vitamin B6 content in yeast samples. Yeast cells were grown in YPD medium with/without 20 μM pyridoxine to OD_{600} of 1.0, and washed with sterile water three times. 7.5 OD_{600} cells were resuspended in 1.35 mL HCl (0.055 N), heat-treated at 121°C for 4 h by using an autoclave (BS-325, Tomy, Tokyo, Japan). After extraction, the pH value was adjusted to 5.0 with 1 N NaOH and the volume was adjusted to 1.5 mL with sterile water, following filtration using a 0.45 μm filter (Sartrius Stedim Biotech, Gottingen, Germany). *S. carlsbergensis* cells were grown on YM plate at 30°C and suspended with sterile distilled water to OD_{600} of 0.2. For bioassay of vitamin B6 content, 100 μL of sample extracts were mixed with 100 μL of the *S. carlsbergensis* cells and 200 μL of vitamin B_6 assay medium (Nissui Pharmaceutical, Tokyo, Japan) in each well of 96 deep-well plate. A standard curve was made using pyridoxine. All test and standard samples were run in triplicate and incubated at 30°C for 20 h with shaking at 150 rpm. A_{595} of 100

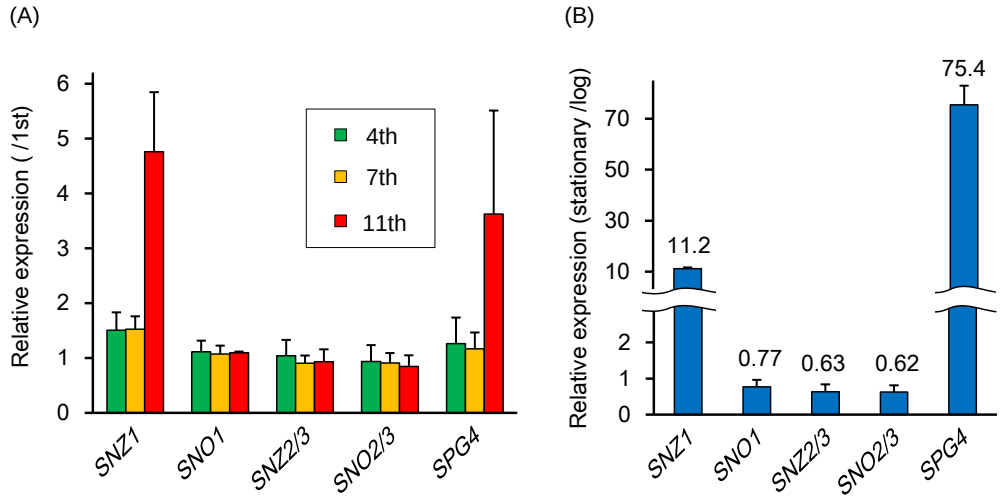

Figure 4.2 RT-qPCR analysis of *SNZ* and *SNO* genes. The expression levels of *SNZ* and *SNO* genes during senescence (A) and during growth to stationary phase (B) were measured by the RT-qPCR method independently for three times. (A) Expression values of designated ages relative to the 1st generation are represented. (B) Cells were grown in YPD media, and then log-phase cells were harvested at OD_{600}=1.0, stationary-phase cells were harvested after 5 days of injection. Expression values relative to expression levels at log phase are shown.

μL of samples was measured using iMark microplate reader (Bio-Rad, Hercules, CA, USA). The total vitamin B6 content in yeast samples was estimated by using the pyridoxine standard curve.

4.3 Results

4.3.1 Transcriptional induction of the SNZ1 gene during senescence

In Chapter 3, microarray analysis for yeast old cells showed that *SNZ1* and *SNO1*, which are involved in PLP biosynthesis, were highly expressed at the 11th generation (Figure 3.4) (81). The *SNZ2* and *SNZ3*, and *SNO2* and *SNO3* genes, which are homologous to *SNZ1* and *SNO1*, respectively, were not induced at the 11th generation. Expression of these genes at the early stage of senescence was confirmed through RT-qPCR analysis. *SNZ1* was highly expressed at the 11th generation, but *SNO1* was constitutively expressed (Figure 4.2A). The *SNZ2*/*SNZ3* and *SNO2*/*SNO3* genes had no change in transcription during senescence. It is reported that these sets of *SNZ* and *SNO* genes are adjacent and coregulated during growth to stationary phase and during nutrient

starvation (54). Upregulation of *SNZ1* during stationary phase was confirmed, but unexpectedly, the *SNO1*, *SNZ2*/*SNZ3* and *SNO2*/*SNO3* genes were not induced during growth to stationary phase (Figure 4.2B). Transcripts of *SPG4* gene, which is known to be upregulated at stationary phase (55), were accumulated at the senescence cells in microarray analysis (Figure 3.4) (81). RT-qPCR analysis verified high expression of *SPG4* in the 11th generation cells (Figure 4.2A) and stationary-phase cells (Figure 4.2B). Thus, age-dependent transcripts accumulation of *SNZ1* and *SPG4*, unlike *SNO1* and other *SNZ* and *SNO*, was observed, suggesting that *SNZ1* and *SPG4* have a specific function of cellular senescence.

4.3.2 Replicative lifespan of age-induced SNZ1 gene deletion mutant

Since *SNZ1* and *SPG4* were the early senescence-induced genes, these genes were possible to be related to cellular aging process and probably to regulate replicative lifespan. To test this possibility, replicative lifespan of the cells deleted for *SNZ1* and *SPG4* was determined. Deletion of *SNZ1* shortened replicative lifespan of approximately 30% compared with the wild-type strain BY4742, but deletion of *SPG4* resulted in a normal replicative lifespan (Figure 4.3A), revealing that *SPG4* is not involved in replicative lifespan. Since Snz1p makes a complex with Sno1p to synthesize PLP, it was examined that whether *SNO1* as well as *SNZ1* regulates replicative lifespan. Deletion of *SNO1* did not decrease lifespan. The Δ*snz2* Δ*snz3* and Δ*sno2* Δ*sno3* double knockout cells were generated and examined for replicative lifespan because the *SNZ2*/*SNZ3* and *SNO2*/*SNO3* genes are highly homologous. No change in lifespan was observed for Δ*snz2* Δ*snz3* and Δ*sno2* Δ*sno3* double mutants (Figure 4.3B). These results indicate that *SNZ1* encoding PLP synthase is a novel longevity gene, suggesting that vitamin B6 synthesis might be important for lifespan regulation.

Since deletion of *SNZ1* shortened replicative lifespan, the effect of overexpression of *SNZ1* on replicative lifespan was examined. The *GAL1p-SNZ1* gene, whose expression is under control of the *GAL1* promoter and induced on galactose media, was constructed and replaced the native *SNZ1* gene on the yeast genome. In the resultant *GAL1p- SNZ1*

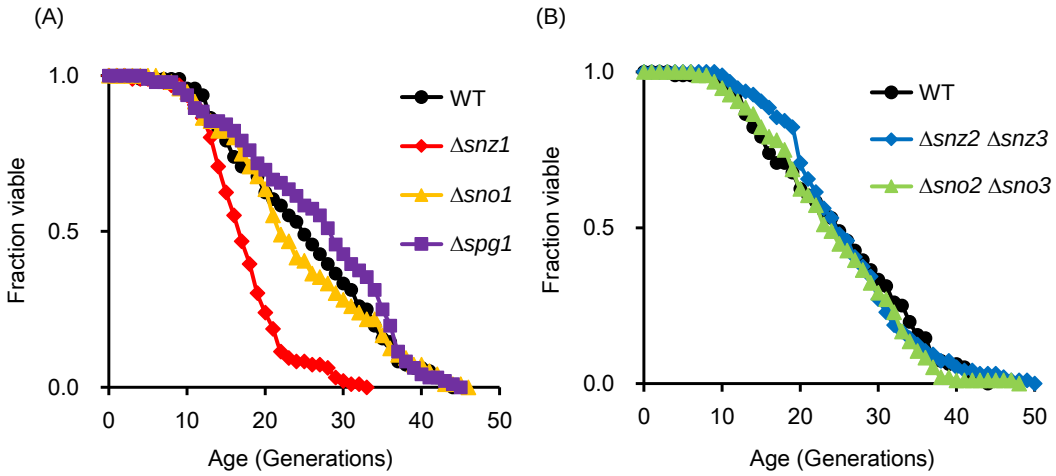

Figure 4.3 Replicative lifespan of vitamin B6 metabolism gene deletion mutants. (A) Lifespan of single deletion mutants of PLP synthase genes was shown. (B) Lifespan of double deletion mutants for *SNZ* and *SNO* genes was shown. Replicative lifespan was measured at least twice for each strain. Average lifespans: BY4742 (wild type, WT), 25.2 generations; Δ*snz1*, 17.5; Δ*sno1*, 24.4; Δ*spg4*, 26.9; Δ*snz2* Δ*snz3*, 26.0; Δ*sno2* Δ*sno3*, 24.5.

strain, transcripts level of *SNZ1* was greatly upregulated after transferring to galactose medium (Figure 4.4A). The mean lifespan of the *GAL1p-SNZ1* cells on galactose medium was 27.0 ± 10.5 generations and the maximal lifespan was 50 generations, while the mean lifespan of the wild-type cells with the *SNZ1* native promoter was 26.7 ± 6.3 and the maximal lifespan 44 generations (Figure 4.4B). Thus, overexpression of *SNZ1* did not extend replicative lifespan.

To clarify the possibility that the age-dependent induction of *SNZ1* is required for maintenance of replicative lifespan, the *PHO4p-SNZ1* gene, in which the *SNZ1* promoter was replaced by constitutively expressed *PHO4* promoter (86), was generated and replaced the native *SNZ1* gene on the genome. In the resultant *PHO4p-SNZ1* strain, *SNZ1* was not upregulated at the 11th generation in comparison to the 1st generation as expectedly (Figure 4.5A). However, the *PHO4p-SNZ1* strain showed normal replicative lifespan (Figure 4.5B). The *SNZ1* transcripts in young *PHO4p-SNZ1* cells increased 6-fold higher than those in young cells having the native *SNZ1* promoter. This higher basal expression of *PHO4p-SNZ1* might cause normal lifespan of the *PHO4p-SNZ1* cells rather than constitutive expression of that gene.

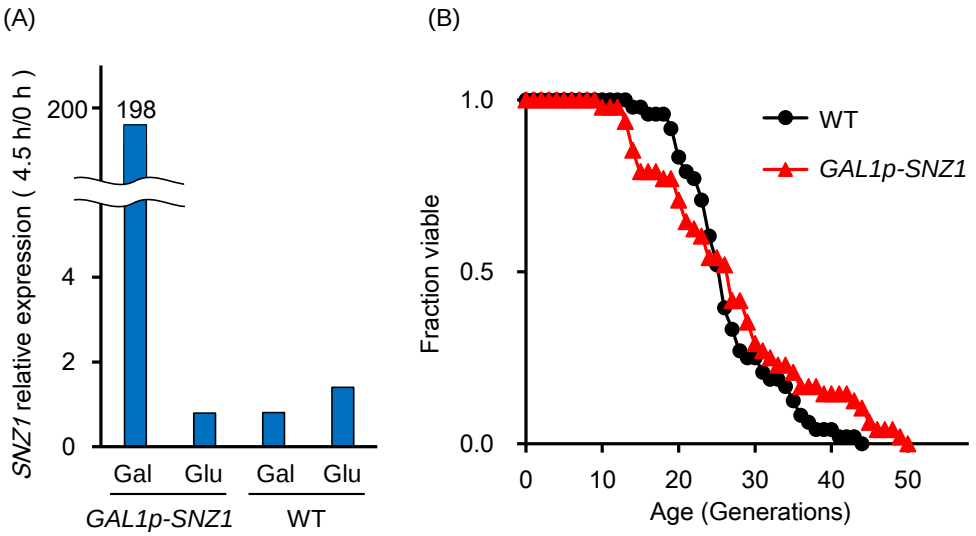

Figure 4.4 Replicative lifespan of *SNZ1*-overexpressed cells. (A) Cells with *SNZ1* from the *GAL1* promoter (*GAL1p-SNZ1*) and with native *SNZ1* promoter (WT) were incubated in galactose or glucose liquid media for 4.5 h. The amount of *SNZ1* mRNA normalized with *UBC6* mRNA was determined with RT-qPCR. Expression values related to transcription levels before induction were shown. (B) Lifespan of the cells with *GAL1p-SNZ1* and wild-type *SNZ1* on galactose medium was shown. Average lifespans: BY4742 (wild type, WT), 26.7 generations; *GAL1p-SNZ1*, 27.0.

Cells with disrupted PLP synthase *SNZ1* gene exhibited short replicative lifespan on YPD plate (containing about 2 μM pyridoxine). Supposing that, when Δ*snz1* cells are cultivated in YPD medium, the intracellular PLP content is not sufficient to maintain their replicative lifespan, excess pyridoxine (20 μM) was supplemented to YPD plate medium and replicative lifespan of Δ*snz1* cells was measured (Figure 4.6A). Interestingly, addition of pyridoxine restored lifespan of Δ*snz1* cells to that of wild-type cells, indicating that intracellular vitamin B6 content was not sufficient for longevity of Δ*snz1* cells. This also suggests that vitamin B6 is necessary for maintenance of replicative lifespan. Addition of excess pyridoxine was expected to also extend replicative lifespan of wild-type cells. However, no significant effect of excess pyridoxine on lifespan of wild-type cells was observed. This did not conflict with the results that overexpression of *SNZ1* did not extend replicative lifespan.

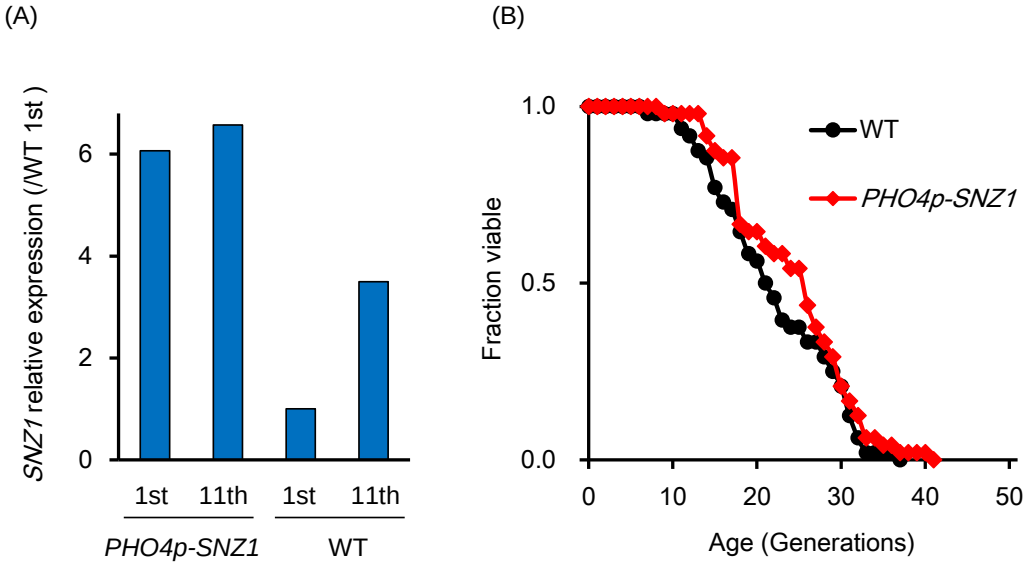

Figure 4.5 Replicative lifespan of *SNZ1*-constitutively expressed cells. (A) Cells with *SNZ1* from the *PHO4* promoter (*PHO4p-SNZ1*) and with native *SNZ1* promoter (WT) at the 11th generation were isolated. The amount of *SNZ1* mRNA normalized with *UBC6* mRNA was determined with RT-qPCR. Expression values related to transcription level in the 1st generation wild-type cells were shown. (B) Lifespan of the cells with *PHO4p-SNZ1* and wild-type *SNZ1* was shown. Average lifespans: BY4742 (wild type, WT), 22.3 generations; *PHO4p-SNZ1*, 24.4.

4.3.3 Replicative lifespan of vitamin B6 uptake gene deletion mutant

Since the replicative lifespan of the cells deleted for PLP synthase *SNZ1* gene was restored by supplementation of excess pyridoxine, intracellular vitamin B6 seemed to regulate replicative lifespan. In *S. cerevisiae*, vitamin B6 (pyridoxine, pyridoxamine and pyridoxal) is transported by Tpn1p, a plasma membrane vitamin B6 transporter (85). To know whether uptake of vitamin B6 is important for longevity, replicative lifespan of cells deleted for *TPN1* was measured on YPD medium. Deletion of *TPN1*, like *SNZ1*, shortened replicative lifespan of approximately 30% compared with wild type (Figure 4.6B), indicating that *TPN1* is also a novel gene that regulates replicative lifespan. Next, effect of addition of pyridoxine to culture media on replicative lifespan of the Δ*tpn1* cells was assessed. Addition of pyridoxine was assumed to have no effect to extend lifespan of Δ*tpn1* cells due to lacking vitamin B6 transporter. However, Δ*tpn1* cells showed normal lifespan on pyridoxine-excess YPD media (Figure 4.6B). These results suggested a passive diffusion of vitamin B6 and/or the existence of another vitamin B6 transporter.

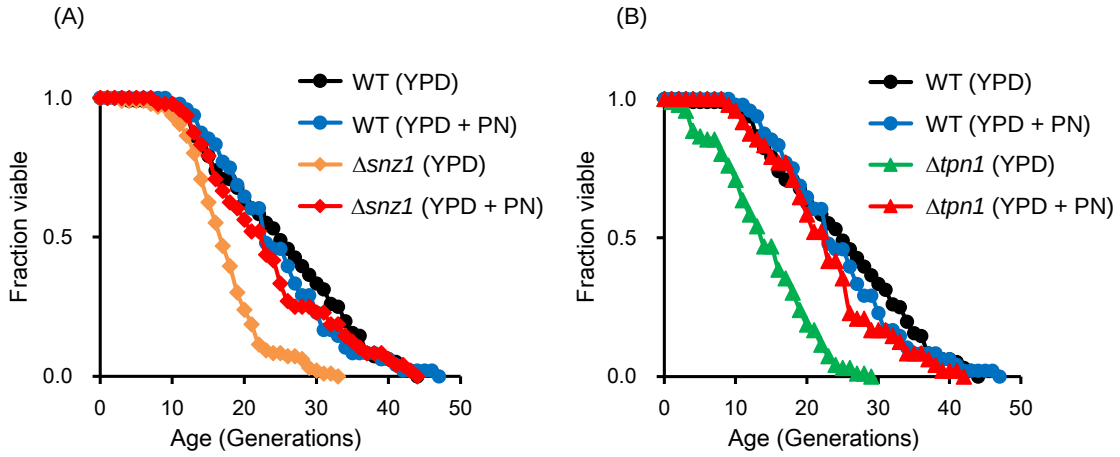

Figure 4.6 (A) Effects of excess pyridoxine supplementation on replicative lifespan. Replicative lifespan of wild-type and Δ*snz1* cells on YPD plate medium supplemented with 20 μM pyridoxine (YPD+PN) was determined. (B) Replicative lifespan of vitamin B6 transporter gene deletion mutant. Replicative lifespan of Δ*tpn1* cells on YPD and YPD+PN plate medium was determined. Average lifespans: BY4742 (wild type, WT) on YPD, 25.2 generations; WT on YPD+PN, 24.6; Δ*snz1* on YPD, 17.5; Δ*snz1* on YPD+PN, 23.4; Δ*tpn1* on YPD, 16.8; Δ*tpn1* on YPD+PN, 22.8.

4.3.4 Intracellular vitamin B6 content in vitamin B6-related gene mutants

Since supplementation of excess pyridoxine restored lifespan of Δ*snz1* and Δ*tpn1* cells, intracellular vitamin B6 in these deletion mutants seemed to be lowered and addition of excess pyridoxine is possible to increase the intracellular contents to the wild-type level. To test this possibility, intracellular vitamin B6 contents were measured by microbiological assay (Figure 4.7). Deletion of the *SNZ1* and *TPN1* gene decreased vitamin B6 contents to 79% and 26%, respectively, compared with wild-type cells. Predictably, supplementation of excess pyridoxine to the media recovered intracellular vitamin B6 contents in Δ*snz1* and Δ*tpn1* cells to that in wild-type cells. Intracellular vitamin B6 contents in wild-type cells were not much higher in the pyridoxine-excess YPD media compared with that in normal YPD media. These results indicated that supplement of pyridoxine extended replicative lifespan of the Δ*snz1* and Δ*tpn1* cells through recovery of intracellular vitamin B6 contents.

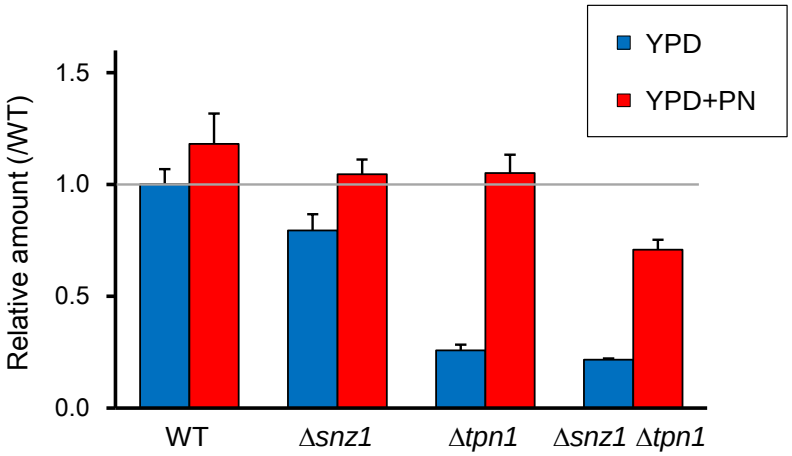

Figure 4.7 Effects of excess pyridoxine supplementation on intracellular vitamin B6 content. The vitamin B6 contents of wild-type, Δ*snz1*, and Δ*tpn1* cells on YPD plate medium supplemented without (YPD) and with 20 μM pyridoxine (YPD+PN) were determined. Relative values of the average amount of vitamin B6 with standard deviations are indicated.

4.3.5 Transcriptional regulation of vitamin B6 synthesis and uptake genes

Since vitamin B6 synthesis *SNZ1* gene was upregulated at the 11th generation, transcription of vitamin B6 uptake *TPN1* gene in the old cells was examined. The microarray analysis for old yeast cells showed that *TPN1* transcripts decreased 40~50% of the 1st generation cells. RT-qPCR analysis confirmed downregulation of the *TPN1* gene in the cells between the 4th and 11th generation approximately 50~70% of the 1st generation cells (Figure 4.8A). Transcription of *TPN1*, unlike *SNZ1*, decreased during senescence rather than was induced.

In senescent cells, *SNZ1* was upregulated and *TPN1* was downregulated. To investigate the possibility that reduced expression of pyridoxine uptake gene causes enhanced expression of pyridoxine biosynthetic genes in old cells, transcriptional level of *SNZ1* gene was measured in the Δ*tpn1* cells (Figure 4.8B). Expression of *SNZ1* in Δ*tpn1* cells was comparable to that in wild-type cells, and similarly expression of *SNO1* did not change in Δ*snz1*, Δ*tpn1*, and wild-type cells. Conversely, deletion of *SNZ1* did not change *TPN1* transcription (Figure 4.8C). Furthermore, overexpression of *SNZ1* by the *GAL1* promoter did not also affect expression of *TPN1* (Figure 4.8D). These results indicated that vitamin B6 biosynthesis and uptake might not interregulate each other.

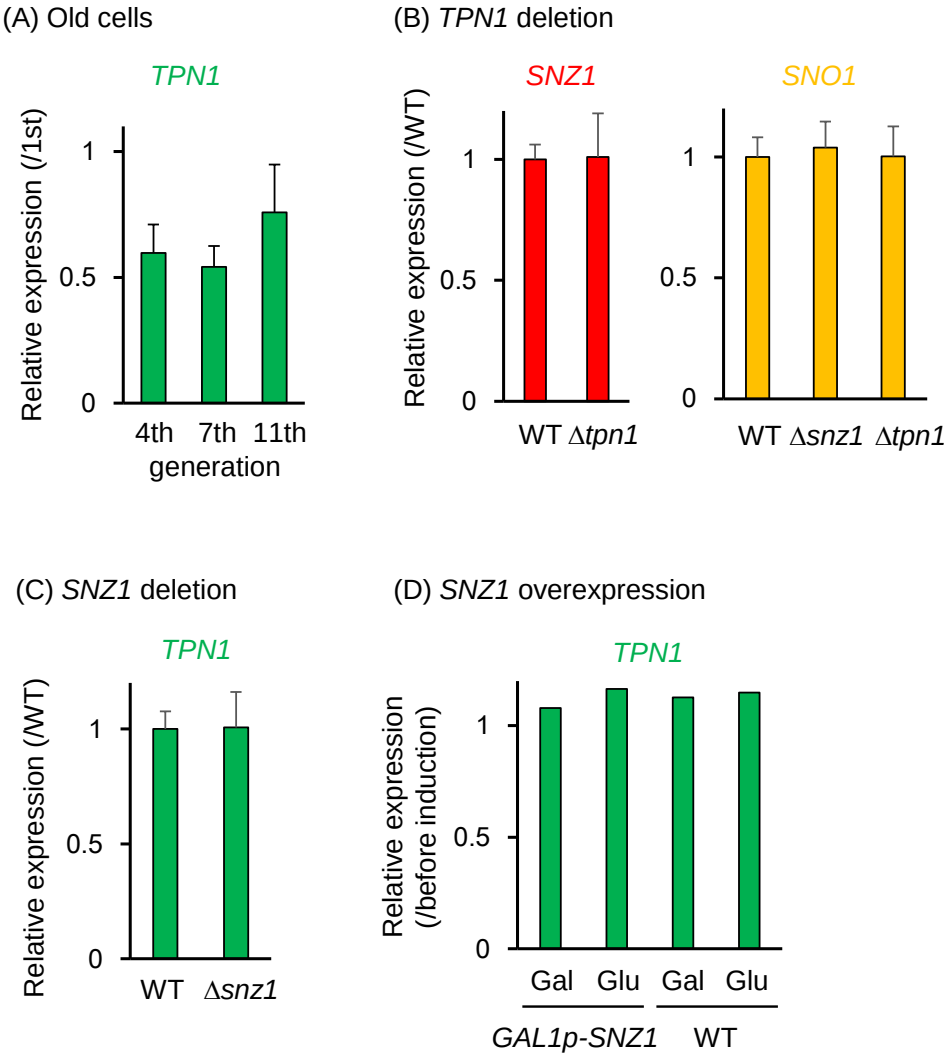

Figure 4.8 Transcriptional analyses of vitamin B6 synthesis and uptake genes. The expression levels were measured by the RT-qPCR method. Relative expression levels of *TPN1* gene during senescence (A), *SNZ1* and *SNO1* genes in Δ*tpn1* cells (B), *TPN1* gene in Δ*snz1* cells (C), were measured independently for three times. (D) *GAL1p-SNZ1* cells were incubated in galactose or glucose liquid media for 4.5 h. The amount of *TPN1* mRNA normalized with *UBC6* mRNA was determined. Expression values related to transcription levels before induction were shown.

4.3.6 Vitamin B6 biosynthesis is required for cell growth

SNZ1, but not *SNO1*, positively regulates replicative lifespan although Snz1p and Sno1p form a complex to catalyze PLP biosynthesis. Since Snz1p and Sno1p seemed to have different function for PLP biosynthesis, requirement of pyridoxine for growth of the *SNZ1*- and *SNO1*-deleted cells was assessed. The Δ*snz1* and Δ*sno1* cells grew normally

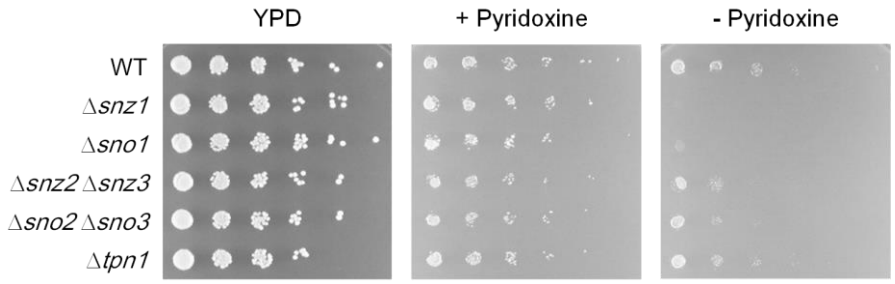

Figure 4.9 Vitamin B6 biosynthesis is required for cell growth. Cells were pre-grown in YPD medium, and then 5-fold serial dilutions were made and spotted onto the YPD, synthetic defined minimal media containing pyridoxine (2 μM) (+Pyridoxine) or without (-Pyridoxine). Images were taken after 2 days of incubation.

on YPD medium as wild-type cells did, but grew extremely slowly in the absence of pyridoxine (Figure 4.9). It was noted that, after 2 days, the Δ*snz1* cells had not grown yet, but the Δ*sno1* cells grew slightly. The Δ*snz2* Δ*snz3* and Δ*sno2* Δ*sno3* double mutants normally grew on pyridoxine-free medium. The Δ*tpn1* cells, which does not import vitamin B6, also grew in the absence of pyridoxine, indicating that biosynthesis of vitamin B6 by Snz1p-Sno1p is sufficient for cell growth. These results reveal that Snz1p had a crucial role in not only biosynthesis of pyridoxine but also regulation of replicative lifespan. This supports the above conclusion that vitamin B6 is essential for replicative lifespan.

4.3.7 Extracellular pyridoxine is not necessary for wild-type longevity

Deletion of vitamin B6 transporter *TPN1* gene was reported to lower intracellular pyridoxine level (87) and in this study shown to shorten replicative lifespan. This led to the idea that depletion of extracellular pyridoxine declines intracellular pyridoxine contents and results in decreasing replicative lifespan. To test this idea, replicative lifespan of wild-type cells was measured on the synthetic complete medium without pyridoxine (Figure 4.10). The mean lifespan on pyridoxine-free medium was 19.3 ± 7.1 generations and the maximal lifespan 37 generations, while the mean lifespan on 2 μM pyridoxine medium (equivalent of contents in YPD media) was 22.8 ± 8.6 generations and the maximal lifespan was 43 generations. This comparable lifespans on between

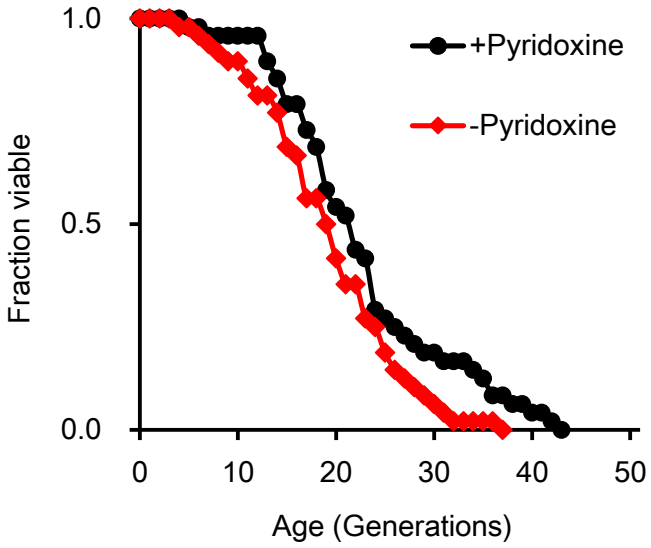

Figure 4.10 Effects of extracellular pyridoxine on replicative lifespan. Replicative lifespan of wild-type cells on synthetic defined minimal media containing pyridoxine (2 μM) (+Pyridoxine) or without (-Pyridoxine) were determined. Average lifespans: on the medium with pyridoxine, 22.8 generations; without pyridoxine, 19.3.

pyridoxine-free and pyridoxine-containing media concluded that biosynthesis of vitamin B6, rather than uptake of vitamin B6, is sufficient for replicative lifespan.

4.3.8 Age-dependent transcription of SNZ1 is induced by Adr1p

Since *SNZ1* transcripts accumulated in the 11th generation cells as well as in the stationary phase, a transcription factor that induces *SNZ1* transcription in old cells was searched. Transcription factors for *SNZ1* expression were predicted using the Yeastract database (http://www.yeastract.com/index.php). Adr1p and Gcn4p binding sites were found in the upstream of the *SNZ1* gene. Adr1p is known to activate genes involved in glucose fermentation (65), glycerol metabolism (88), fatty acid utilization (89), and peroxisome biogenesis (90). Gcn4p is a transcriptional activator of amino acid biosynthetic genes (91). The 11-generation-old cells deleted for *ADR1* or *GCN4* were isolated, and *SNZ1* transcripts in the cells were quantified by RT-qPCR method (Figure 4.11A). In the Δ*adr1* cells, *SNZ1* was not upregulated at the 11th generation. Deletion of *GCN4* did not prevent induction of *SNZ1* expression during senescence although the

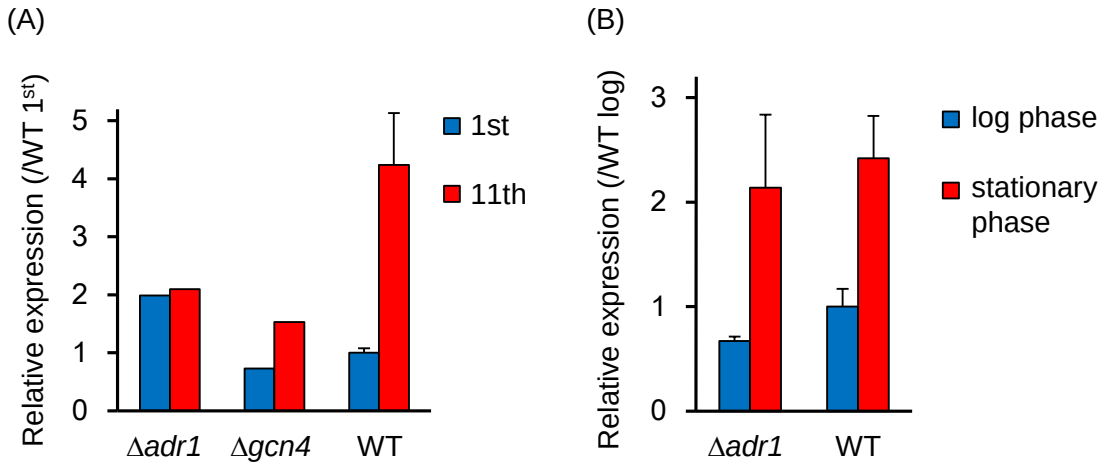

Figure 4.11 Adr1p upregulates transcription of *SNZ1* age-dependently. The *SNZ1* expression levels were measured by the RT-qPCR method. (A) Δ*adr1*, Δ*gcn4*, and wild-type old cells, the amount of *SNZ1* mRNA normalized with *UBC6* mRNA was determined. (B) Relative expression levels of *SNZ1* gene during growth from log phase (OD_{600}=1.0) to stationary phase (after 5 days of injection) in Δ*adr1* and wild-type cells were normalized with *RDN18* transcripts, and measured independently for three times.

expression level of *SNZ1* in Δ*gcn4* cells was relatively lower than wild-type cells. These results reveal that Adr1p is a transcription factor that induces *SNZ1* transcription in old cells.

Next, it was examined that whether Adr1p induces transcription of *SNZ1* in stationary phase. RT-qPCR analysis revealed that *SNZ1* was upregulated during growth to stationary phase in the Δ*adr1* cells as well as in the wild-type cells (Figure 4.11B). This concludes that Adr1p is not a transcription factor that induces *SNZ1* expression in stationary phase.

4.4 Discussion

The transcriptional profiling of aging yeast cells showed that stationary phase-induced *SNZ1* gene were highly expressed at about half the mean lifespan of yeast. Deletion of *SNZ1*, which encodes pyridoxal 5'-phosphate synthase, decreased replicative lifespan. Although Snz1p makes a complex with glutamine amidotransferase Sno1p, *SNO1* was not upregulated during senescence and not related to replicative lifespan. Deletion of *TPN1*, the vitamin B6 transporter gene, decreased replicative lifespan as that

of *SNZ1* did. Supplementation of excess pyridoxine restored replicative lifespan of the Δ*snz1* and Δ*tpn1* cells and also overcame low levels of intracellular vitamin B6 in these cells. These results suggest that vitamin B6 is important for yeast longevity.

Both *SNZ1* and *TPN1* deletion mutants had a similarly short lifespan although intracellular vitamin B6 levels of these cells were largely different. The vitamin B6 content in Δ*tpn1* cells was about 25% of wild type, and that in Δ*snz1* cells was about 80% of wild type. These suggest a threshold of vitamin B6 content that is required to maintain replicative lifespan. When vitamin B6 content was lowered to the threshold, yeast lifespan could be shortened. Alternatively, vitamin B6 contents between the Δ*snz1* and Δ*tpn1* cells might be similarly low in senescent cells although the vitamin B6 contents in this measurement using young cells in logarithmic growth phase were distinctive. That is why deletion of *SNZ1* and *TPN1* showed similarly short lifespan. This is consistent with the idea that transcriptional induction of *SNZ1* during senescence compensates for lack of vitamin B6 in old cells, as described below.

According to the present study, vitamin B6 is necessary for yeast replicative lifespan. Vitamin B6 functions as a cofactor in the form of phosphate ester, such as PLP (84). PLP-dependent enzymes are listed by Percudani and Peracchi, and most of the enzymes are involved in amino acid metabolism (92). 41 PLP-dependent enzymes are found in *S. cerevisiae* (Table 4.2). When intracellular PLP contents are reduced, such as in *tpn1* mutant, the PLP-dependent enzymes could malfunction. Since the Δ*tpn1* cells were replicatively short-lived, it is possible that several of PLP-dependent enzyme genes are involved in replicative lifespan regulation. The 41 PLP-dependent enzyme genes are not reported to positively regulate lifespan, although *UGA1* and *GAD1* genes, which encode GABA metabolic enzymes and are regulated in their activities by PLP, negatively regulate lifespan (93). Whether these PLP-dependent enzyme genes regulate replicative lifespan will be studied.

It has been shown in Chapter 3 that amino acid metabolism declined at the 11th generation cells. This suggests that the activities of PLP-dependent amino acid metabolic enzymes might be declined although transcriptional levels of amino acid biosynthetic enzyme genes were shown to be also reduced. If the enzyme activities decrease, the level

Table 4.2 Yeast PLP-dependent enzymes

Enzyme	Yeast homolog	Description
Aspartate transaminase	*AAT1*	Mitochondrial aspartate aminotransferase; catalyzes the conversion of oxaloacetate to aspartate in aspartate and asparagine biosynthesis
Aspartate transaminase	*AAT2*	Cytosolic aspartate aminotransferase involved in nitrogen metabolism; localizes to peroxisomes in oleate-grown cells
Alanine--glyoxylate transaminase	*AGX1*	Alanine:glyoxylate aminotransferase (AGT); catalyzes the synthesis of glycine from glyoxylate, which is one of three pathways for glycine biosynthesis in yeast
Alanine transaminase	*ALT1*	Alanine transaminase (glutamic pyruvic transaminase); involved in alanine biosynthesis and catabolism; expression is induced in the presence of alanine
Alanine transaminase	*ALT2*	Catalytically inactive alanine transaminase; expression is repressed in the presence of alanine and repression is mediated by Nrg1p; ALT2 has a paralog, ALT1, that arose from the whole genome duplication
Acetylornithine transaminase	*ARG8*	Acetylornithine aminotransferase; catalyzes the fourth step in the biosynthesis of the arginine precursor ornithine
2-aminoadipate transaminase Aromatic-amino-acid transaminase	*ARO8*	Aromatic aminotransferase I; expression is regulated by general control of amino acid biosynthesis
Aromatic-amino-acid transaminase Kynurenine--oxoglutarate transaminase	*ARO9*	Aromatic aminotransferase II; catalyzes the first step of tryptophan, phenylalanine, and tyrosine catabolism
Kynurenine--oxoglutarate transaminase	*BNA3*	Kynurenine aminotransferase; catalyzes formation of kynurenic acid from kynurenine; potential Cdc28p substrate
Branched-chain-amino-acid transaminase	*BAT1*	Mitochondrial branched-chain amino acid (BCAA) aminotransferase; preferentially involved in BCAA biosynthesis; highly expressed during logarithmic phase and repressed during stationary phase
Branched-chain-amino-acid transaminase	*BAT2*	Cytosolic BCAA aminotransferase; preferentially involved in BCAA catabolism; highly expressed during stationary phase and repressed during logarithmic phase
Adenosylmethionine--8-amino-7-oxononanoate transaminase	*BIO3*	7,8-diamino-pelargonic acid aminotransferase (DAPA); catalyzes the second step in the biotin biosynthesis pathway; BIO3 is in a cluster of 3 genes (BIO3, BIO4, and BIO5) that mediate biotin synthesis
Kynureninase	*BNA5*	Kynureninase; required for the de novo biosynthesis of NAD from tryptophan via kynurenine; expression regulated by Hst1p
ornithine-oxo-acid transaminase	*CAR2*	L-ornithine transaminase (OTAse); catalyzes the second step of arginine degradation, expression is dually-regulated by allophanate induction and a specific arginine induction process; not nitrogen catabolite repression sensitive; protein abundance increases in response to DNA replication stress
L-serine ammonia-lyase Threonine ammonia-lyase	*CHA1*	Catabolic L-serine (L-threonine) deaminase; catalyzes the degradation of both L-serine and L-threonine; required to use serine or threonine as the sole nitrogen source, transcriptionally induced by serine and threonine
L-serine ammonia-lyase	*SDL1*	Open reading frame unlikely to produce a functional protein in S288C
Threonine ammonia-lyase	*ILV1*	Threonine deaminase, catalyzes first step in isoleucine biosynthesis; expression is under general amino acid control
Cystathionine gamma-lyase	*CYS3*	Cystathionine gamma-lyase; catalyzes one of the two reactions involved in the transsulfuration pathway that yields cysteine from homocysteine with the intermediary formation of cystathionine; protein abundance increases in response to DNA replication stress
Cystathionine beta-synthase	*CYS4*	Cystathionine beta-synthase; catalyzes synthesis of cystathionine from serine and homocysteine, the first committed step in cysteine biosynthesis; mutations in human ortholog cause homocystinuria
Sphinganine-1-phosphate aldolase	*DPL1*	Dihydrosphingosine phosphate lyase; regulates intracellular levels of sphingolipid long-chain base phosphates (LCBPs), degrades phosphorylated long chain bases, prefers C16 dihydrosphingosine-l-phosphate as a substrate
Glutamate decarboxylase	*GAD1**	Glutamate decarboxylase; converts glutamate into gamma-aminobutyric acid (GABA) during glutamate catabolism; involved in response to oxidative stress
Glycine dehydrogenase (aminomethyl-transferring)	*GCV2*	P subunit of the mitochondrial glycine decarboxylase complex; glycine decarboxylase is required for the catabolism of glycine to 5,10-methylene-THF

Table 4.2 Continued

Enzyme	Yeast homolog	Description
Glycogen phosphorylase	*GPH1*	Glycogen phosphorylase required for the mobilization of glycogen; regulated by cyclic AMP-mediated phosphorylation; expression is regulated by stress-response elements and by the HOG MAP kinase pathway
5-aminolevulinate synthase	*HEM1*	5-aminolevulinate synthase; catalyzes the first step in the heme biosynthetic pathway; an N-terminal signal sequence is required for localization to the mitochondrial matrix; expression is regulated by Hap2p-Hap3p
Histidinol-phosphate transaminase	*HIS5*	Histidinol-phosphate aminotransferase; catalyzes the seventh step in histidine biosynthesis; responsive to general control of amino acid biosynthesis
Cystathionine beta-lyase	*IRC7*	Beta-lyase involved in the production of thiols; expression induced by nitrogen limitation in a *GLN3*, *GAT1*-dependent manner and by copper levels in a Mac1-dependent manner
Cystathionine beta-lyase	*STR3*	Peroxisomal cystathionine beta-lyase; converts cystathionine into homocysteine
Serine C-palmitoyltransferase	*LCB1*	Component of serine palmitoyltransferase; responsible along with Lcb2p for the first committed step in sphingolipid synthesis, which is the condensation of serine with palmitoyl-CoA to form 3-ketosphinganine
Serine C-palmitoyltransferase	*LCB2*	Component of serine palmitoyltransferase; responsible along with Lcb1p for the first committed step in sphingolipid synthesis, which is the condensation of serine with palmitoyl-CoA to form 3-ketosphinganine
Cysteine synthase O-acetylhomoserine aminocarboxypropyltransferase	*MET17*	O-acetyl homoserine-O-acetyl serine sulfhydrylase; required for Methionine and cysteine biosynthesis
Cysteine synthase	*YGR012W*	Putative cysteine synthase; localized to the mitochondrial outer membrane
Phosphoserine transaminase	*SER1*	3-phosphoserine aminotransferase; catalyzes the formation of phosphoserine from 3-phosphohydroxypyruvate, required for serine and glycine biosynthesis; regulated by the general control of amino acid biosynthesis mediated by Gcn4p; protein abundance increases in response to DNA replication stress
Glycine hydroxymethyltransferase	*SHM1*	Mitochondrial serine hydroxymethyltransferase; converts serine to glycine plus 5,10 methylenetetrahydrofolate; involved in generating precursors for purine, pyrimidine, amino acid, and lipid biosynthesis
Glycine hydroxymethyltransferase	*SHM2*	Cytosolic serine hydroxymethyltransferase; converts serine to glycine plus 5,10 methylenetetrahydrofolate; major isoform involved in generating precursors for purine, pyrimidine, amino acid, and lipid biosynthesis
Ornithine decarboxylase	*SPE1*	Ornithine decarboxylase; catalyzes the first step in polyamine biosynthesis; degraded in a proteasome-dependent manner in the presence of excess polyamines; deletion decreases lifespan, and increases necrotic cell death and ROS generation
Cystathionine gamma-synthase	*STR2*	Cystathionine gamma-synthase, converts cysteine into cystathionine
Cystathionine gamma-synthase	*YLL058W*	Putative protein of unknown function with similarity to Str2p; Str2p is a cystathionine gamma-synthase important in sulfur metabolism
Cystathionine gamma-synthase	*YML082W*	Putative protein predicted to have carbon-sulfur lyase activity; transcriptionally regulated by Upc2p via an upstream sterol response element; *YML082W* has a paralog, *STR2*, that arose from the whole genome duplication
Threonine synthase	*THR4*	Threonine synthase; conserved protein that catalyzes formation of threonine from O-phosphohomoserine; expression is regulated by the *GCN4*-mediated general amino acid control pathway
Tryptophan synthase	*TRP5*	Tryptophan synthase; catalyzes the last step of tryptophan biosynthesis; regulated by the general control system of amino acid biosynthesis
4-aminobutyrate--2-oxoglutarate transaminase	*UGA1**	GABA transaminase; also known as 4-aminobutyrate aminotransferase; involved in the 4-aminobutyrate and glutamate degradation pathways; required for normal oxidative stress tolerance and nitrogen utilization; protein abundance increases in response to DNA replication stress

* Deletion of *UGA1* and *GAD1*, unlike *SNZ1* and *TPN1*, extends replicative lifespan (93).

of vitamin B6 may decrease in senescent cells. It is hard to measure vitamin B6 contents in senescent cells because of a small number of old cells (about 5×10^6 cells) prepared by the present procedure. A highly sensitive vitamin B6 assay or a preparation of a large amount of old cells should be developed.

It seems that Adr1p transcription factor promotes *SNZ1* transcription in old cells. This hypothesized that Adr1p transcriptional activation is enhanced by cellular senescence. Although Adr1p is known to be required for carbon source utilization, the carbon source of medium was not reduced when senescent cells were prepared as described in Chapter 3. This indicates that the transcription activity of Adr1p in old cells is not activated by depletion of carbon source. Therefore, signals that regulate Adr1p transcription activity may be independent from between carbon source response and cellular senescence. Although, at present, the aging signal through Adr1p is not clear, Adr1p is an important factor to elucidate the mechanism of cellular senescence and to discover a trigger for cellular senescence.

According to the results of this study, a model of regulation of intracellular vitamin B6 contents by Tpn1p and Snz1p is shown (Figure 4.12). In young cells, Tpn1p mainly supplies vitamin B6 by importing extracellular vitamin B6, and Snz1p less contributes maintenance of vitamin B6 content because the *SNZ1* gene is expressed at low levels in logarithmic growth phase cells as reported previously (54) (Figure 4.12A). In old cells, Snz1p is induced by Adr1p transcriptional activator and largely supplies vitamin B6 by synthesizing PLPs, and Tpn1p is declined by unknown mechanism and less imports vitamin B6 (Figure 4.12B). This model suggests that more vitamin B6 is required for extension of replicative lifespan of the senescent cells. Again, quantification of intracellular PLP in old cells would be required to confirm this model.

4.5 Summary

Transcriptional profiling for aging yeast cells showed that stationary phase-induced genes were highly expressed at about half the median lifespan. Especially, transcriptional level of the *SNZ1* gene, which encodes PLP synthase, greatly increased in old cells. The *SNO1* gene, which encodes glutamine amidotransferase in PLP biosynthesis, was not

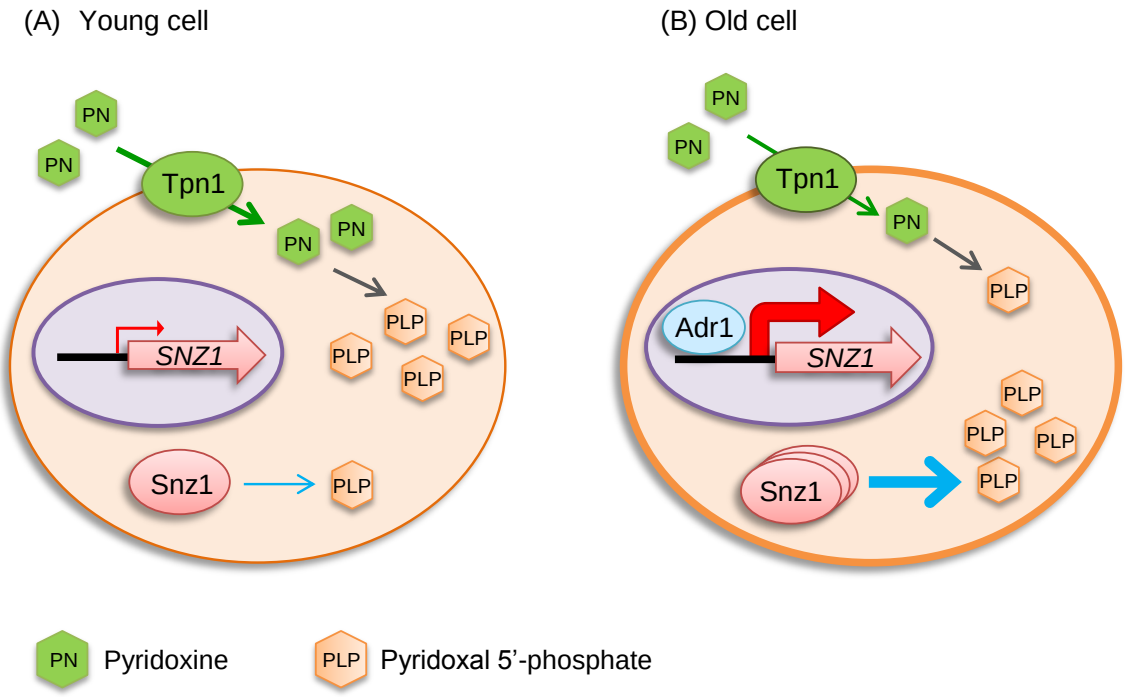

Figure 4.12 Model of regulation of vitamin B6 content by Tpn1p and Snz1p. (A) In young cells, Tpn1p mainly supplies vitamin B6 by importing extracellular vitamin B6. Biosynthesis of vitamin B6 by Snz1p seems to be not much important in modulating vitamin B6 content. (B) In old cells, Snz1p is induced and synthesizes vitamin B6 to contribute regulation of vitamin B6 content.

upregulated with age, although Sno1p forms a complex with Snz1p. Deletion of *SNZ1* decreased replicative lifespan, while overexpression of *SNZ1* did not alter replicative lifespan. Deletions of *SNO1* and the other members of the *SNZ* or *SNO* gene families did not alter replicative lifespan. The Δ*snz1* cells grew extremely slowly, but not the Δ*sno1* cells, in absence of pyridoxine. Addition of excess pyridoxine to culture media restored replicative lifespan of the Δ*snz1* cells. Deletion of vitamin B6 transporter gene *TPN1* shortened replicative lifespan, and replicative lifespan of Δ*tpn1* cells were also restored by supplementation of pyridoxine. These results indicate that vitamin B6 is essential for replicative lifespan.

Chapter 5

Conclusion and general discussion

In this thesis, genes that determine yeast replicative lifespan and factors that regulate cellular senescence were investigated by metabolomic and transcriptomic approaches, and molecular mechanisms for regulation of lifespan and aging were disclosed (Figure 5.1). In Chapter 2, among the target genes that are activated by zinc-finger transcription factor Uga3p, whose deletion extended replicative lifespan as shown previously, *UGA1* as well as *GAD1*, both in the GABA metabolism pathway, was identified as novel aging genes. Lifespan extension by disrupting the *UGA1* or *GAD1* was suggested to be through activating Sir2p function, independently of respiration. In Chapter 3, metabolomic and transcriptomic analyses during the early stage of replicative senescence revealed that, at the 11th generation, amino acid biosynthesis declined, and sugar and TCA cycle metabolism increased, presumably early indications of replicative senescence. Moreover, some of stationary phase-induced genes, including the PLP synthase gene, *SNZ1*, were highly expressed. In Chapter 4, the vitamin B6 biosynthesis (*SNZ1*) and transport (*TPN1*) genes positively regulated replicative lifespan, indicating that vitamin B6 is essential for yeast lifespan.

Previous metabolic fingerprinting of lifespan-related gene deletion mutants showed a correlation between replicative lifespan and metabolic profile (30). Studies in this thesis contribute to supply additional examples that metabolic changes lead to extend or shorten replicative lifespan. Importantly, it is the first case in all organisms that vitamin B6 is involved in lifespan determination. Therefore, more comprehensive metabolome analysis of not only central metabolites but also another metabolites seems to be valuable in identification of novel lifespan-related genes. As a future work, the metabolome analysis using cells deleted for whole metabolic enzyme genes or metabolism-related transcription factor genes needs to be performed.

The investigation of the yeast aging in this thesis is largely informative on the study in the same field in the other eukaryotes including human. The early stage of yeast senescence was fully investigated and, at the 11th generation, about half of average

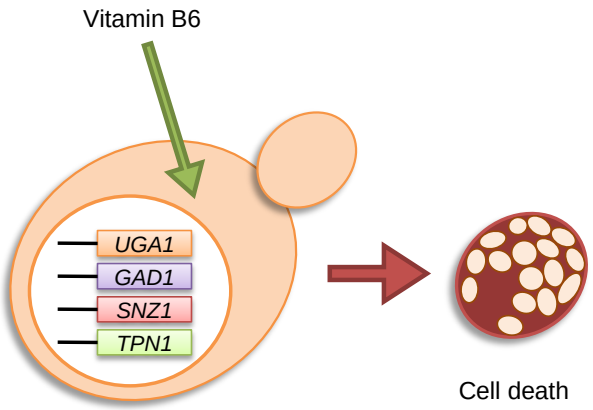

Figure 5.1 Novel lifespan determinants in this thesis. For genetic determinants, four genes were identified: GABA transaminase gene (*UGA1*), glutamate decarboxylase gene (*GAD1*), PLP synthase gene (*SNZ1*), and vitamin B6 transporter gene (*TPN1*). For environmental determinants, vitamin B6 was determined.

lifespan, cellular senescence seemed to begin. In human diploid fibroblasts, the replicative senescence process was divided into four stages (early, middle, advanced, and very advanced) by profiling cellular senescence phenotypes and mRNA expression patterns (16). ROS levels increased in the early stage and were drastically elevated after the middle stage. A low level of SA-β-gal activity was evident in middle stage cells and thereafter gradually increased. Gene expression profiling revealed four distinctive modules: module G1 (doubling times (DT) 2 days), G2 (DT=2~7), G3 (DT=3~20), and G4 (DT=10~30). Gene expression during each module governs each stage of senescence, supporting the development of the associated senescence phenotypes. These findings are consistent with the observation in yeast cells that transcriptional changes of metabolic enzyme genes caused the corresponding metabolic changes. Module G1 was prominently enriched for genes that are related to cell cycle and DNA repair, indicating active cell proliferation. This behavior of human cells is typically similar to that of young yeast cells. Module G2 included genes that are related to metabolic and tRNA processes. Although the correspondence of replicative senescence stages between yeast and human cells is obscure, the 11th generation in yeast cells might correspond to the middle stage of aging of human cells.

In yeast, vitamin B6 is important for replicative lifespan as well as cell growth, and

the levels of vitamin B6 are regulated by vitamin B6 biosynthesis enzyme Snz1p and vitamin B6 transporter Tpn1p. Since animals have no vitamin B6 biosynthesis enzymes, yeast cells that have defects in vitamin B6 synthesis, such as Δ*snz1* cells, can be considered to be a model for animal cells. In human, vitamin B6 transporters have been functionally identified, but their molecular identity has not been determined (94). If the mechanism of regulation of cellular lifespan by vitamin B6 is conserved between yeast and mammals, vitamin B6 transporters could also regulate cellular lifespan in higher eukaryotes, probably their individual lifespan. It is interesting that the human *TPN1* homolog would be identified using yeast mutant cells defective in vitamin B6 biosynthesis.

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Related publications

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Appendix 1 ^{1}H-NMR spectra data in GABA metabolic pathway mutants.

Strain	bin (ppm)	0.000	0.050	0.100	0.150	0.200	0.250	0.300	0.350	0.400	0.450	0.500	0.550	0.600	0.650	0.700	0.751	0.801	0.851	0.901	0.951	1.001	1.051	1.101	1.151	1.201	1.251	1.301	1.351	1.401
WT-1		1.0000	0.0427	0.0047	0.0016	-0.0038	-0.0004	-0.0010	0.0007	0.0017	-0.0015	0.0008	0.0004	0.0007	-0.0045	0.0012	0.0041	0.0278	0.1436	0.2547	0.2940	0.1602	0.0848	0.0794	0.5853	0.3340	0.2211	0.3805	0.2629	0.2879
WT-2		1.0000	0.0850	0.0177	0.0065	0.0006	0.0011	0.0001	0.0004	-0.0009	-0.0005	0.0008	-0.0005	-0.0002	-0.0011	-0.0013	0.0034	0.0295	0.1220	0.2162	0.2498	0.1379	0.0830	0.0743	0.4017	0.2808	0.2070	0.3443	0.2472	0.2527
WT-3		1.0000	0.0306	0.0180	0.0037	0.0040	-0.0018	0.0013	0.0004	-0.0023	0.0023	0.0013	-0.0013	-0.0003	-0.0015	-0.0008	0.0034	0.0402	0.1745	0.2882	0.2994	0.1585	0.0926	0.1151	0.6437	0.3009	0.2583	0.4538	0.2806	0.3268
WT-4		1.0000	0.0218	0.0233	-0.0007	0.0011	0.0018	-0.0007	0.0005	-0.0027	0.0024	-0.0013	0.0007	-0.0020	-0.0012	-0.0019	0.0021	0.0568	0.2230	0.3434	0.3430	0.1759	0.1025	0.1353	0.6328	0.3090	0.3162	0.5391	0.3243	0.3949
uga1-1		1.0000	0.0053	0.0036	-0.0036	-0.0045	0.0033	0.0014	-0.0014	-0.0018	0.0006	-0.0024	0.0014	-0.0028	0.0013	-0.0029	0.0032	0.0391	0.2142	0.3326	0.3280	0.1696	0.0866	0.1060	0.5621	0.2685	0.3287	0.5751	0.3065	0.3792
uga1-2		1.0000	0.0038	0.0028	-0.0007	-0.0057	-0.0002	0.0002	-0.0007	-0.0002	0.0001	0.0005	0.0033	-0.0019	0.0000	0.0001	0.0021	0.0381	0.2098	0.3627	0.3858	0.2038	0.1046	0.1125	0.5731	0.2870	0.2987	0.5734	0.3304	0.4265
uga1-3		1.0000	0.0065	0.0038	-0.0012	-0.0041	0.0004	-0.0021	-0.0005	0.0035	-0.0002	-0.0009	0.0013	-0.0038	-0.0013	-0.0058	0.0029	0.0725	0.2262	0.2741	0.2862	0.1562	0.0872	0.1696	0.4559	0.2150	0.1830	0.3853	0.1679	0.2850
uga1-4		1.0000	0.0056	0.0034	-0.0008	-0.0021	0.0003	0.0016	-0.0015	-0.0023	0.0005	0.0019	0.0011	0.0000	0.0002	-0.0017	0.0055	0.0430	0.2032	0.3118	0.3150	0.1714	0.0928	0.1121	0.5294	0.2902	0.3479	0.5734	0.3238	0.3876
uga2-1		1.0000	0.0218	0.0118	0.0047	0.0001	0.0019	0.0004	-0.0005	0.0002	-0.0005	0.0012	0.0011	-0.0021	-0.0011	0.0036	0.0205	0.0482	0.1668	0.2695	0.2929	0.1589	0.0950	0.1192	0.6056	0.2970	0.2608	0.4505	0.2601	0.3011
uga2-2		1.0000	0.0185	0.0051	0.0005	-0.0033	-0.0005	0.0036	-0.0010	-0.0021	-0.0001	0.0025	-0.0009	-0.0015	-0.0010	-0.0033	0.0092	0.0591	0.2439	0.3715	0.3608	0.1957	0.1147	0.1539	0.7590	0.3662	0.4073	0.6649	0.3818	0.4022
uga2-3		1.0000	0.0283	0.0221	0.0056	-0.0019	0.0013	-0.0012	0.0021	-0.0032	0.0014	0.0005	0.0004	-0.0004	-0.0033	0.0025	0.0138	0.0592	0.2313	0.3617	0.3724	0.1926	0.1113	0.1670	0.8999	0.3883	0.3635	0.6039	0.3495	0.3967
uga2-4		1.0000	0.0298	0.0233	0.0072	-0.0024	0.0019	-0.0006	0.0024	-0.0006	0.0016	-0.0013	0.0024	-0.0026	-0.0012	0.0007	0.0020	0.0602	0.2569	0.3444	0.3247	0.1752	0.1036	0.1246	0.6055	0.3414	0.4471	0.6808	0.3806	0.3720
uga3-1		1.0000	0.0292	0.0135	0.0063	0.0016	-0.0011	0.0008	-0.0014	-0.0001	0.0012	0.0002	-0.0002	0.0015	-0.0028	-0.0003	0.0034	0.0307	0.1501	0.2438	0.2797	0.1561	0.0766	0.1044	0.6430	0.3306	0.2573	0.4086	0.2639	0.3238
uga3-2		1.0000	0.0198	0.0177	0.0043	0.0005	-0.0006	0.0004	-0.0011	0.0014	-0.0003	-0.0007	0.0012	-0.0021	-0.0013	-0.0009	0.0051	0.0376	0.1657	0.2436	0.2550	0.1380	0.0728	0.1061	0.6006	0.2886	0.2951	0.4333	0.2622	0.3182
uga3-3		1.0000	0.0148	0.0039	0.0000	-0.0016	0.0001	-0.0011	0.0003	0.0009	0.0009	-0.0018	-0.0001	0.0013	-0.0013	-0.0009	0.0033	0.0310	0.1377	0.2199	0.2456	0.1328	0.0654	0.0828	0.5355	0.2903	0.2429	0.3844	0.2387	0.2833
uga3-4		1.0000	0.0182	0.0171	0.0087	0.0011	-0.0003	-0.0004	0.0007	-0.0015	0.0007	-0.0002	0.0019	-0.0018	-0.0005	-0.0016	0.0049	0.0279	0.1392	0.2087	0.2179	0.1162	0.0632	0.0906	0.5509	0.2541	0.2473	0.3778	0.2243	0.2688
uga4-1		1.0000	0.0019	0.0034	-0.0026	-0.0032	0.0003	0.0003	0.0006	-0.0015	0.0014	-0.0042	0.0037	-0.0024	0.0009	-0.0068	0.0038	0.0663	0.2028	0.2732	0.3277	0.1739	0.0966	0.1770	1.4310	0.5098	0.2679	0.4349	0.2420	0.3592
uga4-2		1.0000	0.0030	0.0014	-0.0012	-0.0026	0.0006	0.0004	-0.0010	0.0008	0.0025	-0.0037	-0.0017	0.0007	-0.0014	-0.0006	0.0322	0.1156	0.3059	0.4324	0.5197	0.3140	0.2088	0.3037	1.8747	0.6799	0.3810	0.6077	0.3940	0.5351
uga4-3		1.0000	-0.0003	0.0047	-0.0013	0.0002	-0.0007	-0.0002	0.0013	0.0013	-0.0011	0.0018	-0.0022	0.0005	0.0007	-0.0011	0.0039	0.0414	0.1521	0.2248	0.2798	0.1462	0.0919	0.1600	1.0805	0.4421	0.2052	0.3519	0.2050	0.2944
uga4-4		1.0000	0.0005	0.0066	-0.0026	-0.0022	0.0010	-0.0018	0.0006	-0.0025	-0.0001	0.0023	0.0020	-0.0028	0.0002	-0.0006	0.0028	0.0552	0.2020	0.3325	0.4414	0.2406	0.0976	0.1154	1.9901	0.6577	0.2677	0.5186	0.2727	0.4199
gad1-1		1.0000	0.0053	0.0039	-0.0028	-0.0016	-0.0002	-0.0012	-0.0008	0.0013	-0.0009	-0.0011	-0.0008	-0.0007	-0.0050	0.0008	0.0023	0.0438	0.1550	0.2557	0.2486	0.1272	0.0967	0.1722	0.8177	0.3119	0.2358	0.3721	0.2365	0.3197
gad1-2		1.0000	0.0017	0.0032	0.0003	-0.0051	0.0021	0.0023	-0.0030	0.0003	0.0005	-0.0018	0.0030	-0.0009	-0.0027	-0.0015	0.0029	0.0427	0.1915	0.2939	0.3027	0.1568	0.0918	0.1098	0.6213	0.3102	0.3169	0.5015	0.2971	0.3590
gad1-3		1.0000	0.0036	0.0030	-0.0011	-0.0018	-0.0002	0.0007	0.0001	-0.0013	0.0022	-0.0010	0.0004	-0.0004	-0.0026	0.0009	-0.0001	0.0392	0.1894	0.2744	0.2755	0.1418	0.0797	0.0940	0.5376	0.2856	0.3407	0.5233	0.2976	0.3284
gad1-4		1.0000	-0.0018	0.0045	-0.0051	-0.0035	0.0006	-0.0018	0.0014	-0.0040	0.0005	0.0011	-0.0012	0.0007	-0.0029	-0.0047	0.0093	0.0731	0.3183	0.4478	0.4316	0.2226	0.1322	0.1735	1.0085	0.4556	0.5515	0.8101	0.4526	0.5271
uga1gad1-1		1.0000	0.0066	0.0017	-0.0036	-0.0020	-0.0021	0.0026	-0.0031	0.0023	-0.0001	0.0002	0.0010	-0.0023	-0.0019	-0.0001	0.0005	0.0522	0.2293	0.3941	0.4806	0.3207	0.1406	0.1077	0.5501	0.3408	0.4189	0.8403	0.3832	0.4341
uga1gad1-2		1.0000	0.0052	0.0007	-0.0006	-0.0019	0.0016	-0.0003	-0.0013	0.0010	0.0020	-0.0011	0.0005	0.0012	-0.0011	-0.0003	0.0016	0.0391	0.1812	0.3332	0.4242	0.2901	0.1203	0.1051	0.6279	0.3039	0.3117	0.6784	0.2821	0.3798
uga1gad1-3		1.0000	-0.0032	0.0061	-0.0049	0.0008	-0.0021	0.0022	0.0000	-0.0048	0.0024	-0.0004	-0.0021	-0.0007	0.0000	-0.0014	0.0021	0.0446	0.1909	0.3410	0.4296	0.2849	0.1292	0.1139	0.6059	0.3186	0.3434	0.7109	0.3064	0.3916
uga1gad1-4		1.0000	0.0003	0.0042	-0.0005	-0.0041	-0.0007	0.0001	0.0006	0.0014	-0.0012	-0.0017	0.0004	0.0001	-0.0010	-0.0010	0.0015	0.0492	0.1985	0.3420	0.4241	0.2834	0.1265	0.1090	0.5499	0.3091	0.3731	0.7318	0.3182	0.4025

Appendix 1 Continued.

Strain	bin (ppm)	1.451	1.501	1.551	1.601	1.651	1.701	1.751	1.801	1.851	1.901	1.951	2.001	2.051	2.101	2.151	2.201	2.251	2.301	2.351	2.401	2.452	2.502	2.552	2.598	2.649	2.699	2.749	2.799	2.849
WT-1		0.4112	0.2466	0.1265	0.1462	0.2824	0.5113	0.2613	0.1281	0.3581	1.0760	0.2963	0.3404	0.4446	0.4701	0.2897	0.1211	0.1374	0.3336	0.5370	0.1808	0.1318	0.1109	0.1044	0.0777	0.1136	0.0764	0.0649	0.0752	0.0596
WT-2		0.3435	0.2231	0.1173	0.1185	0.2223	0.4028	0.2341	0.1207	0.2745	0.8397	0.2789	0.2896	0.3728	0.3882	0.2591	0.1089	0.1228	0.2624	0.4316	0.1642	0.1118	0.0928	0.0901	0.0610	0.0926	0.0670	0.0485	0.0636	0.0454
WT-3		0.4460	0.2419	0.1308	0.1696	0.3257	0.5533	0.2473	0.1604	0.4487	1.1163	0.2628	0.3842	0.4815	0.4928	0.2800	0.1151	0.1642	0.3935	0.5404	0.1662	0.1380	0.1186	0.1059	0.0758	0.1254	0.0786	0.0614	0.0829	0.0550
WT-4		0.5414	0.2686	0.1489	0.2092	0.4059	0.6485	0.2792	0.2041	0.5748	1.3146	0.3015	0.4691	0.5726	0.5817	0.3143	0.1366	0.2067	0.4867	0.6228	0.1953	0.1584	0.1356	0.1181	0.0839	0.1418	0.0906	0.0715	0.0836	0.0618
uga1-1		0.5804	0.2666	0.1229	0.1858	0.4141	0.7238	0.2601	0.1475	0.5618	1.2491	0.2424	0.3822	0.4627	0.4806	0.2400	0.0585	0.1210	0.3673	0.4955	0.0888	0.0532	0.0332	0.0319	-0.0220	0.0532	-0.0013	-0.0085	0.0100	-0.0425
uga1-2		0.6876	0.3389	0.1660	0.2286	0.4810	0.8560	0.3450	0.1960	0.6375	1.4657	0.3288	0.4713	0.5726	0.5993	0.3455	0.1452	0.2020	0.4746	0.6532	0.1910	0.1537	0.1395	0.1511	0.0906	0.1817	0.1203	0.1097	0.1335	0.0816
uga1-3		0.4970	0.2252	0.1134	0.1769	0.3659	0.6041	0.2599	0.1662	0.4651	1.0546	0.2317	0.3355	0.4473	0.4926	0.2890	0.1025	0.1566	0.3712	0.5150	0.1311	0.0987	0.1000	0.1097	0.0673	0.1476	0.0956	0.0927	0.1101	0.0560
uga1-4		0.5914	0.3066	0.1573	0.2074	0.4124	0.7257	0.3080	0.1774	0.5357	1.2470	0.2893	0.4113	0.4938	0.5155	0.3081	0.1302	0.1862	0.4033	0.5625	0.1769	0.1458	0.1258	0.1345	0.0859	0.1591	0.1102	0.0968	0.1218	0.0764
uga2-1		0.4523	0.2295	0.1304	0.1837	0.3549	0.5557	0.2602	0.1598	0.4281	1.0740	0.2624	0.3563	0.4390	0.4634	0.2724	0.1150	0.1551	0.3608	0.4973	0.1516	0.1287	0.1016	0.0993	0.0657	0.1170	0.0863	0.0738	0.0903	0.0650
uga2-2		0.5827	0.2843	0.1718	0.2476	0.4466	0.6893	0.3108	0.2182	0.5762	1.2942	0.3287	0.4761	0.5750	0.5916	0.3259	0.1485	0.2134	0.4951	0.6305	0.2011	0.1686	0.1333	0.1240	0.0962	0.1546	0.1114	0.1054	0.1233	0.0811
uga2-3		0.5723	0.2618	0.1603	0.2446	0.4202	0.6646	0.2831	0.1889	0.5426	1.3095	0.3086	0.4535	0.5633	0.5880	0.2976	0.1274	0.1946	0.5344	0.5925	0.1853	0.1417	0.1084	0.0994	0.0694	0.1133	0.0940	0.0923	0.1049	0.0628
uga2-4		0.5159	0.2574	0.1577	0.2130	0.3731	0.5684	0.2690	0.1710	0.4654	1.0872	0.2867	0.4234	0.5037	0.5166	0.2877	0.1300	0.1847	0.4197	0.5520	0.1811	0.1469	0.1145	0.1058	0.0782	0.1312	0.0942	0.0906	0.1004	0.0728
uga3-1		0.5526	0.3116	0.1337	0.1799	0.3914	0.7318	0.3270	0.1553	0.4893	1.1932	0.2836	0.3173	0.3908	0.4124	0.2600	0.1032	0.1325	0.3040	0.4518	0.1421	0.1205	0.0966	0.1036	0.0728	0.1003	0.0679	0.0629	0.0899	0.0565
uga3-2		0.5117	0.2725	0.1310	0.1799	0.3735	0.6626	0.2750	0.1528	0.4827	1.0846	0.2467	0.3100	0.3692	0.3847	0.2268	0.0961	0.1323	0.3006	0.3980	0.1291	0.1094	0.0921	0.0949	0.0686	0.0874	0.0601	0.0589	0.0792	0.0498
uga3-3		0.4863	0.2777	0.1187	0.1573	0.3382	0.6467	0.2900	0.1252	0.4200	1.0774	0.2417	0.2802	0.3510	0.3692	0.2340	0.0899	0.1110	0.2673	0.4092	0.1221	0.1054	0.0824	0.0919	0.0562	0.0787	0.0578	0.0514	0.0761	0.0470
uga3-4		0.4370	0.2313	0.1080	0.1501	0.3120	0.5617	0.2328	0.1265	0.4027	0.9150	0.2003	0.2688	0.3199	0.3391	0.2052	0.0853	0.1150	0.2626	0.3589	0.1150	0.1033	0.0817	0.0840	0.0650	0.0774	0.0546	0.0596	0.0794	0.0485
uga4-1		0.6295	0.3036	0.1398	0.2052	0.4544	0.8220	0.3264	0.1818	0.5988	1.3967	0.2776	0.3813	0.4610	0.5093	0.3084	0.1300	0.1834	0.3973	0.5396	0.1714	0.1522	0.1311	0.1309	0.0995	0.1305	0.0842	0.0770	0.0997	0.0694
uga4-2		0.8203	0.4188	0.2251	0.3185	0.6165	1.0280	0.4349	0.2769	0.7858	1.6754	0.3754	0.4929	0.5882	0.6262	0.3843	0.1838	0.2550	0.5085	0.6650	0.2381	0.2097	0.1869	0.1879	0.1583	0.1607	0.1240	0.1219	0.1463	0.1166
uga4-3		0.4940	0.2480	0.1183	0.1675	0.3557	0.6333	0.2770	0.1530	0.4544	1.0934	0.2449	0.3152	0.3775	0.4102	0.2530	0.1090	0.1510	0.3199	0.4327	0.1487	0.1271	0.1066	0.1102	0.0825	0.0950	0.0720	0.0633	0.0847	0.0639
uga4-4		0.7877	0.3811	0.1643	0.2319	0.5315	1.0458	0.4058	0.1707	0.6834	1.7686	0.3400	0.4663	0.5690	0.6441	0.3774	0.1574	0.1978	0.4789	0.6957	0.2183	0.1911	0.1660	0.1681	0.1335	0.1278	0.1091	0.1035	0.1343	0.0878
gad1-1		0.4843	0.2315	0.1261	0.1991	0.3881	0.6041	0.2540	0.2031	0.5512	0.9358	0.2441	0.3636	0.4266	0.4361	0.2504	0.1209	0.1819	0.3944	0.4352	0.1497	0.1289	0.1212	0.1019	0.0782	0.0865	0.0794	0.0896	0.1024	0.0790
gad1-2		0.5779	0.2847	0.1426	0.1988	0.4029	0.7031	0.3008	0.1649	0.5171	1.1931	0.2651	0.3809	0.4680	0.4955	0.3017	0.1207	0.1664	0.3866	0.5300	0.1650	0.1353	0.1166	0.1119	0.0600	0.0838	0.0846	0.0759	0.1005	0.0729
gad1-3		0.5100	0.2559	0.1393	0.1835	0.3535	0.6059	0.2635	0.1506	0.4297	1.0336	0.2370	0.3469	0.4164	0.4378	0.2728	0.1217	0.1572	0.3320	0.4721	0.1546	0.1280	0.1108	0.1031	0.0645	0.0788	0.0782	0.0748	0.0953	0.0684
gad1-4		0.7960	0.3688	0.1880	0.2800	0.5526	0.9498	0.3589	0.2336	0.7388	1.5700	0.3312	0.5356	0.6366	0.6607	0.3603	0.1268	0.2098	0.5372	0.6954	0.1812	0.1446	0.1257	0.1094	0.0364	0.0626	0.0528	0.0590	0.0930	0.0392
uga1gad1-1		0.9573	0.3815	0.1756	0.2633	0.5355	0.8955	0.3957	0.2022	0.6054	1.3495	0.3399	0.4352	0.5557	0.5830	0.3346	0.1393	0.1925	0.4454	0.6559	0.1794	0.1494	0.1289	0.1390	0.0950	0.1642	0.1172	0.1006	0.1303	0.0806
uga1gad1-2		0.8980	0.3202	0.1482	0.2386	0.5123	0.8483	0.3551	0.1820	0.5873	1.2745	0.2864	0.4010	0.5151	0.5542	0.3070	0.1293	0.1847	0.4409	0.6129	0.1668	0.1434	0.1316	0.1365	0.0922	0.1509	0.1124	0.1084	0.1288	0.0743
uga1gad1-3		0.8787	0.3117	0.1534	0.2390	0.4959	0.8090	0.3502	0.1907	0.5676	1.2237	0.2924	0.4144	0.5079	0.5389	0.3051	0.1340	0.1965	0.4303	0.5860	0.1664	0.1410	0.1317	0.1337	0.0961	0.1541	0.1168	0.1018	0.1258	0.0840
uga1gad1-4		0.8960	0.3148	0.1539	0.2463	0.5152	0.8263	0.3504	0.1964	0.5919	1.2184	0.2984	0.4194	0.5176	0.5418	0.3054	0.1355	0.1927	0.4419	0.5930	0.1659	0.1483	0.1329	0.1332	0.0971	0.1562	0.1162	0.1016	0.1345	0.0849

Appendix 1 Continued.

Strain	bin (ppm)	2.899	2.949	2.999	3.049	3.099	3.149	3.199	3.249	3.299	3.349	3.399	3.449	3.499	3.549	3.599	3.649	3.699	3.749	3.799	3.849	3.899	3.949	3.999	4.049	4.099	4.149	4.199	4.249	4.300
WT-1		0.0539	0.0998	0.3872	0.1975	0.1119	0.1409	0.3709	0.4093	0.1363	0.1166	0.1234	0.1539	0.2077	0.3994	0.5271	0.5987	0.3429	0.7865	0.3080	0.2942	0.2284	0.2294	0.1584	0.0667	0.0083	-0.0025	-0.0172	-0.0774	-0.1518
WT-2		0.0392	0.0782	0.2954	0.1747	0.0919	0.1194	0.2663	0.3464	0.1185	0.0938	0.1006	0.1204	0.1639	0.3326	0.3967	0.5004	0.2846	0.6353	0.2762	0.2529	0.1966	0.1933	0.1323	0.0537	0.0006	-0.0044	-0.0231	-0.0747	-0.1344
WT-3		0.0586	0.1213	0.4297	0.1762	0.1222	0.1709	0.4239	0.4028	0.1220	0.1243	0.1404	0.1794	0.2490	0.4617	0.5843	0.6068	0.3891	0.8378	0.2969	0.3156	0.2320	0.2409	0.1462	0.0581	0.0001	-0.0059	-0.0381	-0.1064	-0.1755
WT-4		0.0719	0.1568	0.5185	0.1999	0.1431	0.2090	0.5242	0.4650	0.1403	0.1267	0.1517	0.1919	0.2901	0.5360	0.6630	0.6283	0.4831	0.9871	0.3287	0.3655	0.2772	0.2771	0.1633	0.0518	-0.0035	-0.0096	-0.0364	-0.1308	-0.2122
uga1-1		-0.0223	0.0517	0.5122	0.0936	0.0364	0.0991	0.4928	0.3807	0.0024	-0.0030	0.0375	0.0912	0.2330	0.5491	0.6149	0.5158	0.4037	1.0078	0.2883	0.3173	0.2355	0.2670	0.1628	0.0492	-0.0047	-0.0090	-0.0697	-0.1795	-0.2999
uga1-2		0.0970	0.1771	0.6918	0.2585	0.1699	0.2348	0.6743	0.5776	0.1582	0.1468	0.1956	0.2417	0.3474	0.7101	0.7609	0.6533	0.5067	1.2168	0.3965	0.4262	0.3187	0.3472	0.2088	0.0761	0.0047	-0.0120	-0.0622	-0.1686	-0.2769
uga1-3		0.0769	0.1457	0.4934	0.2090	0.1559	0.1937	0.4943	0.3690	0.0942	0.1111	0.1494	0.2112	0.3581	0.7198	0.7119	0.5666	0.4031	1.0056	0.3233	0.3399	0.2587	0.2911	0.1681	0.0709	0.0003	-0.0066	-0.0339	-0.1126	-0.2027
uga1-4		0.0966	0.1654	0.5923	0.2428	0.1615	0.2125	0.5666	0.5352	0.1520	0.1378	0.1655	0.2048	0.2989	0.6080	0.6593	0.5970	0.4269	1.0241	0.3219	0.3486	0.2658	0.2904	0.1808	0.0725	0.0042	-0.0133	-0.0477	-0.1528	-0.2460
uga2-1		0.0748	0.1258	0.3904	0.1858	0.1160	0.1647	0.4392	0.4663	0.1161	0.1019	0.1219	0.1529	0.2412	0.5171	0.6133	0.6323	0.3633	0.8702	0.2791	0.2840	0.2188	0.2385	0.1472	0.0475	0.0018	-0.0070	-0.0284	-0.0927	-0.1715
uga2-2		0.0970	0.1720	0.5083	0.2191	0.1546	0.2221	0.6043	0.5611	0.1606	0.1555	0.1844	0.2289	0.3529	0.6697	0.7754	0.7010	0.4989	1.0718	0.3507	0.3669	0.2811	0.3083	0.1775	0.0673	0.0010	-0.0099	-0.0412	-0.1243	-0.2295
uga2-3		0.0797	0.1584	0.4825	0.1616	0.1167	0.1838	0.5857	0.5013	0.1359	0.1267	0.1534	0.2085	0.3530	0.6760	0.8343	0.7710	0.4863	1.0881	0.3356	0.3375	0.2772	0.2952	0.1732	0.0509	0.0007	0.0004	-0.0181	-0.0861	-0.1733
uga2-4		0.0842	0.1408	0.4172	0.1873	0.1240	0.1819	0.5090	0.4975	0.1381	0.1373	0.1726	0.2091	0.3081	0.5851	0.6634	0.6170	0.4253	0.9126	0.3165	0.3223	0.2526	0.2730	0.1642	0.0644	0.0032	-0.0087	-0.0352	-0.1125	-0.2102
uga3-1		0.0605	0.1240	0.5523	0.2330	0.1189	0.1735	0.5017	0.5280	0.1297	0.1080	0.1247	0.1514	0.2142	0.4195	0.5434	0.6136	0.3657	0.9160	0.2817	0.2572	0.2034	0.2052	0.1336	0.0541	0.0002	-0.0066	-0.0297	-0.0919	-0.1545
uga3-2		0.0622	0.1247	0.5028	0.1964	0.1078	0.1666	0.4721	0.4804	0.1138	0.0987	0.1183	0.1454	0.2123	0.4009	0.5255	0.5618	0.3546	0.8278	0.2361	0.2487	0.1875	0.1986	0.1217	0.0450	-0.0007	-0.0050	-0.0292	-0.0882	-0.1483
uga3-3		0.0509	0.1042	0.4825	0.2117	0.1017	0.1485	0.4303	0.4823	0.1069	0.0912	0.1082	0.1313	0.1842	0.3685	0.4584	0.5412	0.3090	0.8216	0.2424	0.2294	0.1789	0.1839	0.1191	0.0440	-0.0013	-0.0049	-0.0252	-0.0782	-0.1293
uga3-4		0.0568	0.1049	0.4320	0.1726	0.0998	0.1473	0.4105	0.4159	0.1058	0.0995	0.1188	0.1496	0.2056	0.3775	0.4862	0.5108	0.3124	0.7197	0.2106	0.2135	0.1685	0.1718	0.1076	0.0459	0.0016	-0.0073	-0.0281	-0.0881	-0.1392
uga4-1		0.0883	0.1656	0.6502	0.2468	0.1404	0.2233	0.5710	0.5599	0.1356	0.1400	0.1393	0.1842	0.2637	0.5116	0.9336	0.9621	0.4354	1.0698	0.3016	0.3235	0.2428	0.2744	0.1710	0.0640	-0.0030	0.0017	-0.0396	-0.1268	-0.2205
uga4-2		0.1392	0.2364	0.8109	0.3201	0.2052	0.3017	0.7441	0.6687	0.1863	0.1931	0.1941	0.2448	0.3563	0.6623	1.1799	1.1498	0.5742	1.2939	0.3868	0.4120	0.3042	0.3441	0.2075	0.0719	0.0004	-0.0088	-0.0715	-0.1930	-0.3096
uga4-3		0.0774	0.1356	0.4825	0.2038	0.1168	0.1783	0.4156	0.4623	0.1119	0.1129	0.1124	0.1437	0.2098	0.4211	0.7192	0.8944	0.3488	0.8459	0.2580	0.2794	0.2066	0.2305	0.1440	0.0533	-0.0012	-0.0054	-0.0428	-0.1272	-0.2074
uga4-4		0.1047	0.1766	0.8362	0.2951	0.1665	0.2387	0.7273	0.6749	0.1666	0.1773	0.1787	0.2082	0.2862	0.5838	1.1750	1.1988	0.4783	1.3692	0.3694	0.4064	0.2994	0.3574	0.2167	0.0709	-0.0036	-0.0073	-0.0464	-0.1413	-0.2587
gad1-1		0.1095	0.1986	0.5131	0.2040	0.1681	0.2447	0.5511	0.4298	0.1570	0.1642	0.1924	0.2530	0.3773	0.5818	0.7746	0.6638	0.4725	0.8443	0.2763	0.2900	0.2214	0.2216	0.1484	0.0590	0.0042	-0.0104	-0.0678	-0.1534	-0.2305
gad1-2		0.0896	0.1584	0.5550	0.2253	0.1384	0.2083	0.5686	0.5479	0.1426	0.1398	0.1458	0.1848	0.2754	0.5399	0.6433	0.6174	0.4131	1.0049	0.2890	0.3034	0.2292	0.2435	0.1567	0.0711	0.0044	-0.0086	-0.0518	-0.1390	-0.2218
gad1-3		0.0782	0.1338	0.4719	0.1996	0.1268	0.1780	0.4914	0.4990	0.1303	0.1306	0.1355	0.1704	0.2485	0.4690	0.5487	0.5592	0.3552	0.8738	0.2507	0.2706	0.2110	0.2229	0.1402	0.0533	0.0028	-0.0068	-0.0451	-0.1215	-0.2029
gad1-4		0.0795	0.1759	0.7440	0.2246	0.1526	0.2550	0.8126	0.6460	0.1392	0.1385	0.1531	0.2153	0.3745	0.7338	0.9190	0.7915	0.5674	1.3275	0.3577	0.3908	0.2984	0.3239	0.1850	0.0524	-0.0054	0.0075	-0.0091	-0.1153	-0.2012
uga1gad1-1		0.0834	0.1557	0.6436	0.2646	0.1663	0.2470	0.6959	0.7241	0.1665	0.1436	0.1517	0.2024	0.3121	0.6133	0.5899	0.5579	0.4577	1.1684	0.3478	0.3376	0.2641	0.3339	0.2022	0.0696	-0.0053	-0.0089	-0.0303	-0.1058	-0.2552
uga1gad1-2		0.0858	0.1648	0.6233	0.2426	0.1652	0.2445	0.6616	0.6471	0.1614	0.1496	0.1560	0.1950	0.3120	0.5989	0.6196	0.5860	0.4444	1.1195	0.3106	0.3132	0.2478	0.3003	0.1820	0.0663	-0.0004	-0.0096	-0.0342	-0.1137	-0.2420
uga1gad1-3		0.0901	0.1582	0.5881	0.2408	0.1612	0.2419	0.6315	0.6348	0.1510	0.1422	0.1636	0.2017	0.3126	0.6002	0.6117	0.5813	0.4416	1.0765	0.3202	0.3279	0.2457	0.3212	0.1874	0.0661	-0.0028	-0.0055	-0.0391	-0.1168	-0.2506
uga1gad1-4		0.0977	0.1655	0.6145	0.2606	0.1727	0.2546	0.6612	0.6486	0.1583	0.1464	0.1663	0.2163	0.3282	0.6117	0.6167	0.5498	0.4602	1.0774	0.3113	0.3255	0.2414	0.3128	0.1811	0.0574	-0.0031	-0.0079	-0.0319	-0.1159	-0.2551

Appendix 1 Continued.

Strain	bin (ppm)	4.350	4.400	4.450	4.500	4.550	4.600	4.650	4.700	4.750	4.800	4.850	4.900	4.950	5.000	5.050	5.100	5.150	5.200	5.250	5.300	5.350	5.400	5.450	5.500	5.550	5.600	5.650	5.700	5.750
WT-1		-0.1848	-0.2733	-0.3744	-0.4934	-0.6295	-0.3431	13.5194	141.3079	20.2479	2.9062	1.2260	0.8119	0.6047	0.4830	0.4005	0.3283	0.2735	0.2377	0.2010	0.1685	0.1250	0.1000	0.0755	0.0417	0.0143	-0.0001	-0.0008	-0.0012	0.0013
WT-2		-0.1641	-0.2405	-0.3373	-0.4196	-0.3993	1.5730	10.1775	101.4585	19.2110	4.3948	1.3867	0.7989	0.5712	0.4472	0.3677	0.3036	0.2595	0.2261	0.1875	0.1620	0.1315	0.1056	0.0843	0.0557	0.0314	0.0103	0.0005	-0.0007	0.0011
WT-3		-0.2259	-0.3205	-0.4339	-0.5399	-0.3633	5.4815	21.3620	156.4609	6.4215	2.0087	1.1566	0.8429	0.6466	0.5145	0.4378	0.3558	0.3001	0.2586	0.2168	0.1807	0.1379	0.1112	0.0814	0.0456	0.0124	0.0017	-0.0029	0.0004	0.0014
WT-4		-0.2606	-0.3788	-0.4974	-0.6216	-0.2808	8.5638	28.2332	173.8768	6.0013	2.0917	1.3137	0.9490	0.7302	0.6043	0.5106	0.4348	0.3647	0.3067	0.2668	0.2325	0.1835	0.1534	0.1246	0.0730	0.0416	0.0155	-0.0024	0.0006	0.0007
uga1-1		-0.3877	-0.5433	-0.7421	-0.9372	-0.7087	5.1648	17.7560	151.1663	4.6495	1.6064	1.0154	0.7158	0.5270	0.3785	0.2770	0.1750	0.1061	0.0293	0.0031	0.0010	-0.0005	0.0010	0.0029	-0.0042	-0.0006	-0.0014	0.0052	-0.0031	0.0009
uga1-2		-0.3447	-0.4897	-0.6700	-0.8996	-1.0790	0.7254	16.3865	153.5803	4.3403	1.5672	0.9484	0.6737	0.4759	0.3482	0.2513	0.1702	0.1000	0.0364	-0.0012	0.0060	-0.0065	0.0011	0.0040	-0.0024	0.0037	-0.0032	0.0001	0.0012	-0.0008
uga1-3		-0.2541	-0.3708	-0.5342	-0.6694	-0.7446	4.5722	16.7610	140.9806	3.5924	1.1915	0.7845	0.5641	0.4088	0.3196	0.2507	0.1779	0.1224	0.0770	0.0259	0.0002	-0.0051	0.0030	-0.0010	-0.0006	-0.0004	-0.0013	0.0011	0.0016	0.0021
uga1-4		-0.3275	-0.4599	-0.6335	-0.8133	-0.9662	2.3271	13.9959	138.3343	4.3251	1.5376	0.9157	0.6498	0.4623	0.3381	0.2450	0.1552	0.0936	0.0208	0.0033	0.0177	-0.0095	0.0030	0.0010	-0.0047	0.0006	-0.0006	0.0010	-0.0011	-0.0022
uga2-1		-0.2095	-0.3091	-0.4073	-0.4867	-0.4238	4.0002	15.7781	120.5448	5.1650	1.7206	1.0109	0.7105	0.5530	0.4456	0.3716	0.3080	0.2588	0.2128	0.1742	0.1495	0.1071	0.0741	0.0466	0.0145	0.0023	0.0017	-0.0024	-0.0012	0.0020
uga2-2		-0.2824	-0.4127	-0.5663	-0.6996	-0.2488	7.6230	25.0439	170.0887	6.2758	2.1845	1.3890	0.9989	0.7701	0.6224	0.5189	0.4362	0.3694	0.3148	0.2685	0.2324	0.1733	0.1282	0.0937	0.0519	0.0175	-0.0026	0.0006	0.0023	-0.0017
uga2-3		-0.2113	-0.3144	-0.4229	-0.5410	-0.6447	-0.6865	0.7366	11.8022	131.1067	127.8257	3.6829	1.7505	1.1701	0.8824	0.6962	0.5670	0.4737	0.4011	0.3362	0.2964	0.2341	0.1886	0.1369	0.0985	0.0680	0.0360	0.0067	-0.0027	0.0021
uga2-4		-0.2623	-0.3781	-0.5116	-0.6597	-0.5607	4.7275	20.5262	154.0097	5.9747	2.0977	1.2366	0.8974	0.6788	0.5415	0.4525	0.3736	0.3056	0.2614	0.2241	0.1943	0.1338	0.1005	0.0556	0.0184	-0.0005	-0.0030	0.0015	-0.0006	0.0008
uga3-1		-0.1921	-0.2720	-0.3710	-0.4772	-0.4626	2.1923	14.1760	115.9999	9.7291	1.8270	0.9503	0.6805	0.5085	0.3999	0.3377	0.2777	0.2298	0.1912	0.1574	0.1365	0.0985	0.0692	0.0413	0.0137	-0.0024	0.0021	-0.0033	-0.0008	0.0001
uga3-2		-0.1831	-0.2660	-0.3568	-0.4455	-0.4018	3.8248	15.9475	110.1448	4.2269	1.3987	0.8693	0.6192	0.4775	0.3877	0.3289	0.2756	0.2300	0.1951	0.1692	0.1566	0.1134	0.0825	0.0590	0.0330	0.0116	-0.0005	0.0010	-0.0027	0.0012
uga3-3		-0.1619	-0.2358	-0.3173	-0.4027	-0.4186	1.3368	12.1425	107.9956	6.7824	1.3888	0.8042	0.5673	0.4292	0.3444	0.2837	0.2318	0.1978	0.1637	0.1327	0.1214	0.0834	0.0605	0.0399	0.0082	0.0011	0.0013	-0.0005	-0.0012	0.0015
uga3-4		-0.1776	-0.2457	-0.3376	-0.4079	-0.3807	3.0594	15.0311	110.5804	3.8255	1.2788	0.7905	0.5666	0.4396	0.3461	0.2940	0.2407	0.2050	0.1646	0.1397	0.1242	0.0828	0.0590	0.0324	0.0093	0.0023	0.0008	-0.0002	0.0004	-0.0022
uga4-1		-0.2667	-0.3846	-0.5399	-0.6745	-0.7605	3.0149	18.7074	172.5817	5.2329	1.7248	1.0190	0.7727	0.6128	0.4960	0.4169	0.3443	0.2836	0.2366	0.1993	0.1629	0.1174	0.0879	0.0578	0.0198	0.0014	-0.0031	0.0004	0.0002	-0.0001
uga4-2		-0.4035	-0.5816	-0.7759	-0.9435	-0.1142	8.0283	24.1250	198.2734	5.6993	1.9751	1.1962	0.8695	0.6213	0.4755	0.3703	0.2521	0.1888	0.1089	0.0356	0.0057	-0.0020	0.0052	0.0100	-0.0053	-0.0005	-0.0013	0.0011	-0.0023	-0.0005
uga4-3		-0.2436	-0.3505	-0.4645	-0.5820	-0.4441	3.9890	15.3265	130.8354	4.7163	1.4878	0.8189	0.5666	0.4040	0.2937	0.2087	0.1441	0.0882	0.0291	-0.0022	0.0041	-0.0081	-0.0084	0.0005	-0.0060	0.0021	0.0003	0.0011	-0.0020	0.0013
uga4-4		-0.2991	-0.4458	-0.6216	-0.8526	-1.1432	-1.4060	11.6682	212.9753	6.1695	2.0938	1.3054	0.9615	0.7275	0.5942	0.4973	0.4134	0.3496	0.2890	0.2387	0.1879	0.1380	0.1018	0.0697	0.0156	0.0024	0.0005	-0.0036	-0.0027	0.0007
gad1-1		-0.2991	-0.4318	-0.5424	-0.5952	0.8485	9.6676	28.7190	180.5563	5.2967	1.7152	0.9997	0.6632	0.4630	0.3354	0.2448	0.1619	0.0863	0.0294	-0.0009	0.0081	-0.0076	-0.0019	-0.0003	-0.0009	-0.0011	0.0005	-0.0014	0.0000	-0.0021
gad1-2		-0.2956	-0.4047	-0.5682	-0.7409	-0.8742	1.6249	16.3861	151.2336	5.2097	1.7242	0.8926	0.6218	0.4392	0.3080	0.2286	0.1487	0.0902	0.0290	0.0014	0.0059	-0.0084	-0.0023	0.0019	-0.0036	-0.0004	0.0002	-0.0006	0.0031	-0.0010
gad1-3		-0.2687	-0.3803	-0.5145	-0.6808	-0.7947	1.3359	12.5158	136.7895	4.4948	1.5039	0.8183	0.5791	0.3893	0.2865	0.2060	0.1322	0.0708	0.0228	0.0011	0.0074	-0.0015	-0.0093	-0.0042	-0.0070	0.0016	-0.0004	-0.0015	0.0030	-0.0008
gad1-4		-0.2795	-0.4111	-0.5671	-0.6703	-0.1556	8.4578	25.7476	8.2892	5.4053	1.5463	0.8023	0.5469	0.3838	0.2564	0.1821	0.0894	0.0303	-0.0062	0.0026	0.0140	0.0013	-0.0005	0.0006	-0.0033	0.0022	-0.0017	0.0008	-0.0025	0.0014
uga1gad1-1		-0.3232	-0.4666	-0.6136	-0.7962	-0.6977	4.5098	15.9631	147.5816	9.6889	1.8522	0.9498	0.6541	0.4509	0.3265	0.2295	0.1463	0.0867	0.0220	-0.0009	0.0127	0.0003	-0.0013	-0.0004	0.0000	0.0014	-0.0031	0.0029	-0.0006	-0.0024
uga1gad1-2		-0.3031	-0.4177	-0.5759	-0.7337	-0.7428	4.3193	17.2018	150.4733	4.7693	1.5768	0.8741	0.6001	0.4319	0.3042	0.2239	0.1356	0.0861	0.0265	-0.0014	0.0081	-0.0059	0.0030	-0.0011	0.0002	0.0006	-0.0015	0.0005	-0.0023	0.0025
uga1gad1-3		-0.3131	-0.4454	-0.5963	-0.7446	-0.5110	4.9934	17.3113	144.6868	4.5948	1.5787	0.8906	0.6281	0.4462	0.3156	0.2293	0.1498	0.0868	0.0276	-0.0022	0.0118	-0.0067	-0.0005	-0.0005	-0.0005	0.0023	-0.0024	0.0005	-0.0003	0.0031
uga1gad1-4		-0.3279	-0.4529	-0.5948	-0.7431	-0.3408	5.9146	18.6730	152.6694	4.9611	1.5899	0.9103	0.6317	0.4484	0.3292	0.2431	0.1506	0.0803	0.0260	0.0014	0.0073	-0.0011	-0.0016	-0.0013	-0.0012	0.0016	-0.0014	0.0001	0.0000	0.0014

Appendix 1 Continued.

Strain	bin (ppm)	5.800	5.850	5.900	5.951	6.001	6.051	6.101	6.151	6.201	6.251	6.301	6.351	6.401	6.451	6.501	6.551	6.601	6.651	6.701	6.751	6.801	6.851	6.901	6.951	7.001	7.051	7.101	7.151	7.201
WT-1		-0.0009	-0.0011	0.0001	0.0039	-0.0025	0.0020	0.0040	-0.0007	-0.0010	0.0001	-0.0008	-0.0001	-0.0005	-0.0013	0.0013	0.0011	-0.0016	-0.0009	0.0000	-0.0011	0.0014	0.0003	0.0017	-0.0029	-0.0007	-0.0006	0.0122	0.0032	-0.0027
WT-2		-0.0015	-0.0020	0.0000	0.0037	-0.0016	-0.0001	0.0029	-0.0031	-0.0025	-0.0012	-0.0003	0.0014	-0.0006	-0.0003	0.0012	-0.0007	-0.0004	-0.0006	0.0022	-0.0028	0.0009	0.0015	-0.0001	-0.0014	-0.0006	-0.0003	0.0099	0.0021	-0.0011
WT-3		-0.0013	0.0000	-0.0001	0.0057	-0.0047	0.0014	0.0036	-0.0013	-0.0023	-0.0004	0.0017	-0.0034	0.0020	0.0006	-0.0006	-0.0004	0.0005	0.0002	0.0007	-0.0014	-0.0021	0.0043	-0.0005	-0.0024	-0.0007	-0.0009	0.0140	0.0022	-0.0023
WT-4		-0.0006	0.0004	-0.0001	0.0112	-0.0030	0.0013	0.0040	-0.0013	-0.0022	0.0017	-0.0023	0.0021	-0.0005	0.0006	0.0004	-0.0004	-0.0010	-0.0020	0.0034	-0.0032	0.0036	-0.0008	0.0020	-0.0052	0.0025	-0.0040	0.0239	0.0018	-0.0027
uga1-1		0.0005	0.0018	-0.0037	0.0073	0.0005	0.0021	0.0036	-0.0028	-0.0016	-0.0021	-0.0004	0.0007	0.0006	0.0009	0.0014	-0.0011	0.0005	-0.0016	0.0026	-0.0040	-0.0007	0.0034	-0.0031	0.0032	-0.0001	-0.0024	0.0302	0.0023	-0.0013
uga1-2		-0.0013	-0.0036	0.0035	0.0060	-0.0042	0.0020	0.0055	-0.0016	-0.0027	0.0009	0.0014	-0.0006	-0.0011	-0.0002	0.0023	-0.0046	-0.0014	0.0020	-0.0016	-0.0036	0.0043	-0.0010	0.0035	-0.0024	0.0003	-0.0059	0.0302	0.0062	-0.0007
uga1-3		-0.0033	-0.0006	-0.0005	0.0108	-0.0037	-0.0016	0.0057	-0.0021	-0.0028	-0.0015	-0.0002	-0.0006	0.0025	-0.0010	0.0000	0.0005	-0.0015	-0.0005	-0.0019	-0.0002	0.0025	-0.0023	0.0014	0.0001	-0.0002	-0.0054	0.0318	0.0042	-0.0026
uga1-4		-0.0004	0.0026	-0.0016	0.0034	-0.0037	0.0023	0.0043	-0.0037	0.0002	0.0011	-0.0006	-0.0009	0.0002	0.0009	-0.0021	0.0034	-0.0018	-0.0008	0.0014	-0.0001	0.0013	0.0016	0.0005	-0.0016	-0.0023	0.0000	0.0340	0.0043	-0.0036
uga2-1		-0.0024	-0.0015	0.0003	0.0053	-0.0040	0.0020	0.0042	-0.0011	-0.0047	0.0012	0.0008	-0.0012	-0.0003	0.0009	-0.0018	0.0015	-0.0001	-0.0018	0.0004	-0.0010	-0.0006	0.0006	0.0015	-0.0014	-0.0020	-0.0023	0.0244	0.0044	-0.0024
uga2-2		0.0000	-0.0014	-0.0001	0.0047	-0.0017	-0.0009	0.0073	-0.0011	-0.0010	-0.0022	0.0001	0.0008	-0.0023	0.0011	0.0019	-0.0033	0.0030	-0.0001	0.0004	-0.0014	-0.0021	0.0020	0.0008	0.0022	-0.0040	-0.0011	0.0279	0.0026	-0.0027
uga2-3		0.0001	-0.0037	-0.0035	0.0069	-0.0003	0.0019	0.0012	-0.0037	0.0014	-0.0012	0.0017	0.0019	-0.0001	0.0000	0.0017	-0.0020	0.0015	-0.0007	-0.0010	-0.0002	-0.0008	0.0005	0.0010	-0.0039	-0.0034	0.0016	0.0064	0.0290	-0.0052
uga2-4		-0.0009	-0.0024	-0.0010	0.0072	0.0010	-0.0035	0.0053	0.0009	-0.0043	0.0014	0.0004	-0.0003	-0.0011	0.0010	-0.0004	-0.0012	0.0019	-0.0006	-0.0023	-0.0036	0.0037	-0.0001	0.0006	-0.0028	0.0003	-0.0013	0.0182	0.0033	-0.0003
uga3-1		0.0017	-0.0005	0.0002	0.0010	-0.0007	0.0005	0.0037	-0.0023	-0.0006	-0.0002	-0.0009	0.0011	-0.0008	-0.0008	-0.0001	0.0010	-0.0004	-0.0004	0.0013	-0.0018	-0.0014	0.0016	0.0006	-0.0006	-0.0013	-0.0014	0.0233	0.0091	0.0010
uga3-2		-0.0008	0.0005	-0.0013	0.0033	-0.0045	0.0017	0.0042	-0.0033	-0.0024	0.0033	0.0002	0.0014	-0.0047	0.0030	0.0009	-0.0015	0.0009	0.0001	-0.0002	-0.0012	-0.0007	0.0015	0.0013	-0.0036	-0.0010	-0.0002	0.0198	0.0060	-0.0022
uga3-3		-0.0030	-0.0005	0.0008	0.0016	-0.0025	-0.0001	0.0038	-0.0003	-0.0020	-0.0006	-0.0002	0.0008	0.0007	-0.0010	0.0007	0.0006	-0.0007	0.0013	0.0004	-0.0026	-0.0005	0.0011	0.0026	-0.0016	-0.0015	0.0003	0.0202	0.0093	0.0012
uga3-4		0.0029	-0.0034	-0.0006	0.0018	-0.0005	0.0013	0.0007	-0.0006	0.0004	-0.0014	-0.0005	-0.0007	0.0026	0.0014	-0.0025	0.0006	0.0012	-0.0011	0.0011	-0.0013	-0.0008	0.0024	-0.0013	-0.0016	-0.0011	0.0001	0.0197	0.0039	0.0014
uga4-1		0.0006	-0.0006	-0.0007	0.0050	0.0000	-0.0025	0.0041	0.0017	-0.0007	-0.0020	0.0001	0.0022	-0.0017	-0.0012	-0.0004	0.0016	-0.0014	0.0016	-0.0025	-0.0013	0.0008	-0.0008	0.0036	-0.0015	0.0010	-0.0033	0.0300	0.0049	-0.0033
uga4-2		0.0013	0.0006	-0.0023	0.0067	-0.0043	0.0030	0.0063	-0.0020	-0.0012	0.0001	0.0002	0.0008	-0.0040	0.0011	-0.0001	-0.0028	0.0038	0.0006	-0.0021	-0.0006	-0.0028	0.0033	0.0011	-0.0024	0.0003	-0.0021	0.0295	0.0083	-0.0036
uga4-3		-0.0009	-0.0030	0.0005	0.0035	-0.0028	0.0024	0.0010	-0.0012	-0.0029	0.0010	0.0005	-0.0008	-0.0001	-0.0007	0.0000	0.0016	-0.0011	0.0000	0.0005	-0.0003	0.0000	0.0001	0.0035	-0.0033	0.0003	-0.0033	0.0252	0.0070	-0.0029
uga4-4		0.0004	0.0021	-0.0041	0.0057	-0.0015	-0.0013	0.0093	0.0000	-0.0035	0.0000	0.0021	-0.0011	-0.0017	-0.0003	0.0006	-0.0009	0.0002	0.0045	-0.0018	-0.0023	-0.0015	0.0036	0.0032	-0.0022	-0.0003	-0.0036	0.0382	0.0079	-0.0038
gad1-1		0.0006	-0.0034	0.0020	0.0010	0.0026	0.0011	0.0010	0.0011	0.0007	-0.0008	-0.0027	0.0012	0.0014	0.0027	0.0012	-0.0017	0.0034	-0.0022	-0.0013	0.0025	-0.0018	0.0019	0.0019	0.0004	-0.0042	-0.0034	0.0082	-0.0020	-0.0012
gad1-2		0.0003	-0.0021	0.0008	0.0043	-0.0020	0.0020	0.0028	-0.0026	-0.0004	0.0005	0.0002	0.0012	-0.0005	-0.0003	-0.0002	-0.0008	0.0018	0.0000	0.0002	0.0005	0.0005	-0.0008	0.0018	-0.0013	-0.0015	-0.0007	0.0247	0.0076	-0.0056
gad1-3		-0.0019	-0.0023	0.0012	0.0041	-0.0048	0.0035	-0.0003	0.0023	-0.0025	0.0010	0.0008	-0.0006	0.0010	-0.0024	0.0029	-0.0023	0.0015	-0.0013	-0.0002	-0.0002	0.0009	-0.0003	0.0029	0.0001	-0.0032	-0.0006	0.0227	0.0025	-0.0002
gad1-4		-0.0013	-0.0064	-0.0012	0.0100	-0.0070	-0.0003	0.0052	0.0011	-0.0058	-0.0001	0.0052	-0.0016	-0.0024	0.0016	-0.0013	0.0037	-0.0023	0.0018	-0.0018	-0.0005	-0.0018	-0.0016	0.0011	0.0003	-0.0006	-0.0031	0.0391	0.0100	-0.0096
uga1gad1-1		0.0004	-0.0002	-0.0041	0.0045	-0.0030	0.0001	0.0033	-0.0005	-0.0007	-0.0019	-0.0035	0.0024	-0.0015	0.0008	0.0016	0.0000	0.0001	-0.0027	-0.0009	0.0026	-0.0030	0.0029	0.0001	0.0000	-0.0063	0.0006	0.0507	0.0116	-0.0022
uga1gad1-2		-0.0002	-0.0038	0.0038	0.0072	-0.0056	0.0009	0.0044	0.0021	-0.0020	-0.0007	0.0007	-0.0008	-0.0014	0.0008	-0.0023	0.0026	-0.0021	-0.0009	0.0016	0.0025	-0.0050	0.0027	0.0000	-0.0031	-0.0004	-0.0007	0.0507	0.0078	-0.0042
uga1gad1-3		-0.0025	-0.0039	0.0036	0.0021	-0.0029	-0.0006	0.0066	-0.0017	-0.0021	0.0015	0.0007	-0.0028	0.0014	0.0001	0.0013	-0.0045	0.0009	0.0016	0.0002	-0.0002	-0.0018	-0.0013	0.0033	-0.0066	-0.0034	0.0021	0.0473	0.0054	-0.0047
uga1gad1-4		0.0003	-0.0020	-0.0022	0.0054	-0.0032	0.0001	0.0026	0.0003	-0.0012	-0.0014	-0.0007	-0.0014	0.0005	-0.0015	0.0009	0.0019	-0.0027	0.0012	0.0001	0.0007	-0.0041	0.0024	0.0019	-0.0026	-0.0049	0.0001	0.0523	0.0062	-0.0040

Appendix 1 Continued.

Strain	bin (ppm)	7.251	7.301	7.351	7.401	7.451	7.501	7.551	7.602	7.652	7.702	7.752	7.798	7.849	7.899	7.949	7.999	8.049	8.099	8.149	8.199	8.249	8.299	8.349	8.399	8.449	8.499	8.549	8.599	8.649
WT-1		0.0019	0.0218	0.0201	0.0130	-0.0027	0.0009	-0.0013	0.0009	-0.0009	0.0002	0.0010	-0.0001	-0.0020	0.0018	0.0106	0.0121	0.0046	-0.0046	0.0001	0.0014	0.0020	-0.0018	-0.0023	0.0029	-0.0024	-0.0017	0.0043	-0.0029	0.0006
WT-2		0.0002	0.0189	0.0161	0.0162	-0.0013	0.0026	-0.0019	-0.0014	0.0017	0.0010	-0.0003	-0.0005	-0.0009	0.0003	0.0076	0.0106	0.0043	-0.0036	-0.0005	0.0001	0.0032	-0.0014	-0.0011	0.0006	-0.0015	-0.0007	0.0033	-0.0036	0.0005
WT-3		0.0004	0.0280	0.0196	0.0167	0.0002	-0.0031	0.0022	0.0016	-0.0042	0.0015	0.0010	-0.0020	0.0004	-0.0010	0.0111	0.0110	0.0083	-0.0095	0.0036	-0.0008	0.0044	-0.0039	-0.0035	0.0033	0.0003	-0.0036	0.0055	-0.0031	-0.0018
WT-4		0.0008	0.0315	0.0265	0.0177	-0.0013	0.0016	-0.0035	0.0019	0.0007	-0.0017	0.0011	-0.0010	0.0007	-0.0027	0.0176	0.0137	0.0018	-0.0039	0.0128	0.0036	0.0097	0.0005	-0.0017	0.0035	-0.0018	-0.0019	0.0057	-0.0025	-0.0015
uga1-1		-0.0053	0.0119	0.0008	0.0064	-0.0084	-0.0013	0.0016	0.0006	0.0002	0.0010	0.0015	-0.0019	-0.0025	-0.0029	0.0209	0.0223	0.0012	-0.0029	0.0035	-0.0004	0.0029	-0.0023	-0.0048	0.0069	0.0012	-0.0057	0.0047	0.0013	-0.0058
uga1-2		-0.0036	0.0296	0.0060	0.0101	-0.0078	-0.0009	0.0001	0.0036	-0.0013	0.0010	0.0001	-0.0035	0.0020	-0.0034	0.0252	0.0202	0.0021	-0.0047	0.0064	-0.0043	0.0046	-0.0024	-0.0024	0.0008	0.0022	-0.0054	0.0062	-0.0046	-0.0024
uga1-3		-0.0025	0.0245	0.0044	0.0072	-0.0091	0.0041	0.0007	-0.0015	0.0001	-0.0013	0.0016	0.0012	-0.0006	-0.0021	0.0251	0.0165	0.0038	-0.0028	0.0005	0.0019	0.0023	-0.0017	-0.0006	0.0021	-0.0013	-0.0028	0.0044	-0.0025	0.0004
uga1-4		-0.0042	0.0104	-0.0021	0.0040	0.0006	-0.0009	-0.0017	0.0005	-0.0006	-0.0028	0.0026	-0.0011	-0.0007	-0.0025	0.0237	0.0194	-0.0001	-0.0060	0.0040	-0.0026	0.0038	-0.0023	-0.0029	0.0025	-0.0005	-0.0042	0.0052	-0.0010	-0.0049
uga2-1		0.0003	0.0268	0.0169	0.0195	-0.0023	0.0014	-0.0024	0.0012	-0.0009	-0.0003	-0.0006	0.0001	-0.0028	0.0006	0.0164	0.0205	0.0001	-0.0033	0.0020	-0.0001	0.0059	-0.0029	0.0003	0.0009	0.0010	-0.0012	0.0045	-0.0030	0.0006
uga2-2		-0.0001	0.0345	0.0262	0.0235	-0.0027	-0.0009	-0.0022	-0.0007	0.0005	0.0042	-0.0061	0.0031	0.0014	-0.0037	0.0247	0.0163	0.0009	-0.0023	0.0015	-0.0031	0.0060	-0.0004	-0.0028	0.0029	-0.0014	-0.0033	0.0051	-0.0052	-0.0001
uga2-3		0.0031	0.0430	0.0265	0.0230	-0.0021	0.0014	-0.0008	0.0005	-0.0027	0.0047	-0.0024	0.0008	-0.0034	-0.0027	0.0065	0.0230	0.0148	-0.0075	0.0101	0.0027	0.0022	0.0017	-0.0036	0.0072	-0.0013	-0.0033	0.0088	-0.0076	0.0020
uga2-4		-0.0028	0.0231	0.0018	0.0062	-0.0064	0.0008	-0.0008	-0.0003	0.0011	-0.0019	0.0025	-0.0045	-0.0005	-0.0020	0.0244	0.0122	0.0050	-0.0091	0.0034	0.0000	0.0058	-0.0016	-0.0032	0.0023	0.0011	-0.0041	0.0037	-0.0032	-0.0011
uga3-1		-0.0024	0.0284	0.0192	0.0211	0.0006	0.0012	-0.0021	0.0001	0.0001	0.0011	0.0013	-0.0040	0.0011	-0.0012	0.0169	0.0188	0.0001	-0.0045	0.0041	0.0007	0.0000	0.0006	-0.0010	0.0006	-0.0008	-0.0018	0.0034	-0.0016	-0.0018
uga3-2		0.0014	0.0259	0.0176	0.0197	-0.0048	0.0018	-0.0013	0.0017	-0.0008	0.0007	-0.0007	0.0002	0.0004	-0.0010	0.0143	0.0159	0.0043	-0.0048	0.0008	-0.0008	0.0027	-0.0007	-0.0020	0.0003	-0.0014	0.0015	0.0002	0.0007	-0.0010
uga3-3		-0.0022	0.0108	0.0028	0.0035	-0.0037	-0.0001	-0.0016	0.0009	0.0008	-0.0004	0.0002	0.0011	-0.0023	0.0009	0.0165	0.0180	0.0023	-0.0030	0.0003	0.0017	0.0024	-0.0016	-0.0003	0.0008	-0.0009	-0.0027	0.0043	-0.0019	0.0001
uga3-4		-0.0021	0.0184	0.0095	0.0075	-0.0041	0.0043	-0.0039	0.0010	-0.0010	-0.0011	-0.0005	0.0007	-0.0007	-0.0013	0.0168	0.0105	-0.0006	-0.0011	0.0012	0.0010	0.0023	-0.0018	-0.0033	0.0011	0.0001	-0.0009	0.0032	-0.0018	-0.0030
uga4-1		0.0005	0.0320	0.0200	0.0197	-0.0019	0.0004	0.0011	-0.0023	0.0011	0.0007	-0.0006	-0.0013	-0.0005	-0.0009	0.0123	0.0335	0.0055	-0.0067	0.0020	-0.0012	0.0019	-0.0017	0.0005	0.0008	-0.0022	-0.0022	0.0034	-0.0021	-0.0031
uga4-2		-0.0010	0.0300	0.0066	0.0098	-0.0108	0.0023	-0.0015	-0.0010	-0.0024	0.0003	-0.0018	-0.0001	-0.0023	0.0015	0.0244	0.0323	0.0000	-0.0034	0.0024	-0.0025	0.0083	-0.0050	-0.0016	-0.0007	0.0010	0.0021	-0.0005	-0.0022	0.0010
uga4-3		-0.0026	0.0304	0.0180	0.0196	-0.0005	0.0012	-0.0022	0.0011	-0.0001	-0.0013	0.0020	-0.0017	-0.0010	-0.0005	0.0073	0.0224	0.0013	-0.0021	0.0027	-0.0019	0.0036	0.0004	-0.0009	0.0012	-0.0030	-0.0006	0.0024	-0.0007	-0.0010
uga4-4		0.0009	0.0470	0.0258	0.0360	-0.0020	0.0004	-0.0013	-0.0001	-0.0008	0.0014	-0.0019	-0.0043	0.0019	-0.0008	0.0250	0.0336	0.0062	-0.0062	0.0038	-0.0036	0.0069	-0.0025	-0.0051	0.0035	-0.0012	-0.0049	0.0071	-0.0059	0.0021
gad1-1		-0.0068	0.0095	-0.0017	0.0025	-0.0044	-0.0018	0.0003	-0.0007	-0.0007	0.0022	-0.0022	-0.0019	0.0018	-0.0019	0.0125	0.0014	-0.0036	-0.0015	0.0022	-0.0007	-0.0006	-0.0025	0.0004	0.0020	-0.0028	0.0013	0.0014	-0.0010	-0.0008
gad1-2		-0.0017	0.0140	-0.0036	0.0062	-0.0031	-0.0029	-0.0012	0.0014	0.0005	-0.0017	-0.0001	-0.0015	0.0003	-0.0003	0.0090	0.0223	0.0037	-0.0051	0.0029	0.0000	0.0046	-0.0022	-0.0009	0.0015	-0.0022	-0.0052	0.0075	-0.0046	0.0041
gad1-3		-0.0026	0.0197	0.0037	0.0062	-0.0019	-0.0041	0.0004	0.0020	-0.0011	0.0014	-0.0003	-0.0006	0.0001	0.0006	0.0085	0.0205	-0.0001	-0.0041	0.0004	-0.0001	0.0036	-0.0043	0.0009	-0.0009	-0.0001	-0.0036	0.0019	-0.0012	-0.0018
gad1-4		-0.0066	0.0168	-0.0039	0.0080	-0.0124	0.0047	-0.0037	-0.0016	0.0016	-0.0003	0.0015	-0.0032	-0.0002	0.0016	0.0205	0.0262	0.0042	-0.0070	0.0066	-0.0025	0.0008	-0.0005	-0.0027	-0.0031	0.0018	-0.0025	0.0008	-0.0004	-0.0011
uga1gad1-1		-0.0113	0.0182	-0.0044	0.0085	-0.0078	-0.0031	-0.0001	0.0026	-0.0036	0.0029	-0.0018	0.0012	-0.0005	-0.0038	0.0409	0.0531	0.0041	-0.0052	0.0026	-0.0011	0.0073	-0.0029	-0.0015	0.0041	-0.0034	0.0000	0.0011	0.0014	-0.0011
uga1gad1-2		-0.0111	0.0149	-0.0037	0.0101	-0.0052	-0.0011	0.0001	0.0018	-0.0031	0.0017	-0.0007	-0.0038	0.0004	-0.0022	0.0306	0.0436	0.0046	-0.0041	0.0024	-0.0051	0.0087	-0.0060	-0.0014	0.0021	-0.0034	0.0002	-0.0010	0.0002	-0.0029
uga1gad1-3		0.0013	0.0383	0.0198	0.0234	-0.0015	0.0014	0.0015	-0.0002	0.0011	-0.0016	0.0016	-0.0024	-0.0018	-0.0008	0.0362	0.0452	-0.0003	-0.0012	0.0011	-0.0035	0.0096	-0.0055	0.0014	0.0012	-0.0018	-0.0030	0.0040	-0.0030	0.0001
uga1gad1-4		-0.0021	0.0393	0.0204	0.0219	-0.0026	0.0029	-0.0022	-0.0005	0.0043	-0.0028	0.0035	-0.0041	-0.0008	0.0008	0.0346	0.0435	0.0034	-0.0022	0.0031	-0.0052	0.0085	-0.0047	0.0000	0.0020	-0.0017	-0.0036	0.0061	-0.0025	-0.0005

Appendix 1 Continued.

Strain	bin (ppm)	8.699	8.749	8.799	8.849	8.899	8.949	8.999	9.049	9.099	9.149	9.199	9.249	9.299	9.349	9.399	9.449	9.500	9.550	9.600	9.650	9.700	9.750	9.800	9.850	9.900	9.9497
WT-1		-0.0014	-0.0001	-0.0006	0.0010	-0.0020	0.0016	-0.0023	0.0012	-0.0028	0.0031	-0.0013	-0.0021	0.0037	0.0004	-0.0004	0.0002	-0.0014	0.0010	-0.0008	0.0007	-0.0008	0.0017	-0.0022	0.0015	-0.0001	-0.0015
WT-2		0.0004	-0.0021	0.0008	0.0015	0.0012	-0.0010	-0.0016	0.0000	-0.0030	0.0033	-0.0012	-0.0012	0.0020	0.0001	0.0020	-0.0032	0.0007	-0.0003	0.0003	-0.0012	0.0004	-0.0001	-0.0012	0.0011	0.0000	-0.0017
WT-3		0.0019	-0.0022	-0.0016	0.0027	-0.0003	-0.0011	-0.0001	-0.0003	-0.0002	0.0000	-0.0031	0.0009	0.0046	-0.0027	-0.0011	-0.0014	0.0010	0.0004	-0.0023	0.0006	-0.0020	0.0012	-0.0004	-0.0005	-0.0030	0.0027
WT-4		0.0020	-0.0042	0.0031	-0.0002	0.0005	-0.0029	-0.0001	-0.0009	-0.0004	0.0063	-0.0025	-0.0006	0.0064	-0.0025	-0.0035	0.0039	-0.0034	0.0022	-0.0041	0.0017	0.0014	-0.0018	-0.0019	0.0032	-0.0008	0.0003
uga1-1		0.0047	-0.0016	0.0004	0.0034	-0.0042	0.0000	0.0013	-0.0013	-0.0018	0.0039	0.0000	-0.0044	0.0044	-0.0009	-0.0020	0.0034	-0.0034	0.0019	-0.0010	0.0029	-0.0014	0.0017	-0.0024	0.0033	0.0003	-0.0043
uga1-2		-0.0016	-0.0016	0.0013	-0.0029	-0.0009	-0.0025	0.0043	-0.0029	-0.0043	0.0071	-0.0031	-0.0019	0.0035	0.0017	-0.0020	-0.0009	0.0033	-0.0014	0.0010	0.0012	-0.0002	-0.0002	-0.0029	0.0010	-0.0009	0.0021
uga1-3		0.0009	-0.0032	0.0029	-0.0024	0.0013	-0.0012	0.0015	-0.0010	-0.0013	0.0050	-0.0042	-0.0005	0.0023	0.0012	-0.0010	0.0012	-0.0043	0.0041	-0.0019	0.0015	-0.0011	-0.0009	-0.0007	-0.0007	0.0008	-0.0021
uga1-4		0.0050	-0.0047	0.0033	-0.0018	0.0031	-0.0020	0.0002	-0.0027	-0.0004	0.0007	-0.0020	-0.0013	0.0029	-0.0007	-0.0004	-0.0028	-0.0004	-0.0032	0.0029	0.0008	-0.0011	-0.0032	-0.0002	0.0011	0.0018	-0.0008
uga2-1		0.0003	-0.0036	0.0042	-0.0015	0.0003	0.0005	-0.0010	-0.0017	-0.0006	0.0043	-0.0030	-0.0009	0.0010	0.0003	-0.0001	-0.0016	-0.0006	0.0010	-0.0025	0.0028	0.0001	-0.0024	0.0016	-0.0006	-0.0012	-0.0001
uga2-2		-0.0018	-0.0008	0.0027	0.0010	-0.0022	-0.0003	-0.0015	0.0011	-0.0024	0.0045	-0.0032	-0.0014	0.0048	-0.0036	0.0029	-0.0031	-0.0012	0.0029	-0.0013	-0.0001	0.0000	-0.0009	0.0020	-0.0036	-0.0003	0.0041
uga2-3		-0.0013	-0.0051	0.0042	-0.0026	0.0012	0.0003	-0.0005	-0.0044	0.0082	-0.0009	-0.0020	-0.0014	0.0037	-0.0005	-0.0021	-0.0014	-0.0001	-0.0010	0.0011	0.0005	-0.0038	-0.0010	0.0017	-0.0007	0.0000	0.0000
uga2-4		0.0036	-0.0043	0.0033	-0.0034	0.0015	0.0015	-0.0022	-0.0002	-0.0025	0.0061	-0.0050	-0.0002	0.0025	-0.0009	-0.0015	-0.0004	0.0001	0.0001	0.0018	-0.0005	-0.0002	-0.0029	0.0039	-0.0017	-0.0018	0.0021
uga3-1		0.0009	-0.0021	0.0017	0.0009	-0.0011	0.0001	-0.0022	-0.0020	0.0025	0.0020	-0.0013	-0.0026	0.0020	-0.0011	-0.0011	0.0006	-0.0026	0.0030	-0.0013	0.0016	-0.0009	-0.0015	0.0049	-0.0031	-0.0010	0.0010
uga3-2		-0.0006	-0.0014	0.0018	-0.0010	-0.0001	0.0018	-0.0020	-0.0001	-0.0013	0.0025	-0.0008	-0.0026	0.0029	-0.0013	-0.0009	0.0006	0.0015	-0.0017	0.0001	-0.0010	0.0030	-0.0020	0.0005	-0.0012	0.0006	-0.0007
uga3-3		-0.0014	0.0000	0.0005	-0.0015	0.0008	-0.0005	-0.0007	0.0016	-0.0034	0.0037	-0.0013	-0.0011	0.0030	-0.0015	-0.0012	0.0020	-0.0023	0.0001	0.0013	0.0003	-0.0014	0.0012	-0.0005	-0.0001	-0.0004	-0.0007
uga3-4		0.0020	-0.0002	-0.0009	0.0024	-0.0020	0.0018	-0.0002	-0.0015	-0.0024	0.0040	-0.0004	-0.0029	0.0023	-0.0005	-0.0020	0.0019	-0.0008	0.0002	-0.0012	-0.0002	0.0005	0.0016	-0.0012	0.0004	-0.0015	0.0010
uga4-1		0.0009	-0.0016	0.0021	0.0019	-0.0036	0.0029	-0.0023	-0.0015	-0.0003	0.0020	-0.0007	-0.0013	0.0019	-0.0012	0.0007	-0.0002	0.0000	0.0021	0.0007	-0.0035	-0.0018	0.0017	-0.0002	0.0012	-0.0024	0.0032
uga4-2		0.0020	-0.0030	0.0030	0.0004	-0.0019	0.0009	-0.0013	-0.0011	-0.0006	0.0058	-0.0031	-0.0024	0.0047	-0.0017	0.0002	-0.0011	-0.0014	-0.0005	0.0019	-0.0006	-0.0006	0.0011	0.0017	-0.0040	0.0009	0.0015
uga4-3		0.0012	-0.0013	0.0029	-0.0017	0.0016	-0.0003	-0.0007	-0.0014	-0.0012	0.0042	-0.0044	-0.0005	0.0033	-0.0017	0.0008	0.0000	-0.0025	0.0019	0.0002	-0.0009	-0.0006	0.0012	-0.0021	0.0005	-0.0002	0.0000
uga4-4		-0.0028	0.0000	0.0009	0.0003	0.0017	-0.0053	0.0035	0.0013	-0.0037	0.0033	0.0002	-0.0016	0.0032	-0.0018	-0.0013	-0.0010	-0.0015	-0.0004	0.0002	0.0000	0.0013	-0.0016	-0.0009	0.0001	0.0001	0.0002
gad1-1		-0.0014	0.0031	0.0002	0.0002	0.0024	-0.0017	-0.0014	-0.0016	0.0032	0.0001	-0.0009	-0.0019	0.0016	0.0005	-0.0025	0.0024	0.0011	-0.0015	0.0019	-0.0022	0.0005	0.0006	0.0022	-0.0028	0.0019	-0.0019
gad1-2		-0.0024	0.0010	0.0010	-0.0013	-0.0010	-0.0007	0.0018	-0.0020	-0.0003	0.0034	-0.0041	-0.0016	0.0041	0.0003	-0.0022	0.0014	-0.0017	0.0013	-0.0003	0.0009	0.0022	-0.0007	-0.0010	0.0017	-0.0013	0.0013
gad1-3		-0.0016	0.0040	-0.0021	0.0016	-0.0001	-0.0011	-0.0016	0.0002	-0.0015	0.0003	-0.0007	-0.0009	0.0031	-0.0006	-0.0008	0.0004	-0.0003	0.0003	-0.0011	0.0004	-0.0021	0.0014	-0.0001	-0.0024	0.0024	-0.0003
gad1-4		0.0008	-0.0013	0.0005	-0.0008	0.0034	-0.0032	-0.0036	0.0037	-0.0049	0.0031	-0.0037	0.0015	0.0049	-0.0012	-0.0010	-0.0011	0.0010	-0.0008	-0.0018	0.0028	0.0008	0.0000	0.0018	-0.0026	-0.0035	0.0052
uga1gad1-1		-0.0002	-0.0016	0.0018	0.0009	0.0014	0.0007	-0.0001	-0.0030	0.0024	-0.0006	-0.0028	0.0026	0.0029	-0.0046	0.0025	0.0001	-0.0043	0.0016	-0.0010	0.0001	0.0009	0.0010	-0.0013	0.0010	0.0025	-0.0022
uga1gad1-2		0.0011	-0.0029	0.0035	-0.0023	-0.0010	0.0004	-0.0028	0.0015	-0.0032	0.0025	-0.0011	-0.0002	0.0044	-0.0014	0.0002	0.0001	0.0014	-0.0017	0.0020	-0.0036	0.0033	-0.0024	-0.0007	-0.0005	0.0016	-0.0032
uga1gad1-3		-0.0008	0.0004	-0.0010	0.0031	-0.0037	0.0008	-0.0009	0.0002	-0.0009	0.0005	-0.0024	-0.0008	0.0047	-0.0022	-0.0003	-0.0001	-0.0027	0.0012	0.0012	0.0006	0.0004	-0.0036	0.0012	0.0021	0.0012	-0.0028
uga1gad1-4		-0.0020	0.0021	-0.0017	0.0015	-0.0003	-0.0024	0.0004	-0.0020	0.0012	0.0026	-0.0007	-0.0001	0.0019	-0.0005	-0.0011	-0.0014	0.0024	-0.0005	-0.0008	0.0007	-0.0009	0.0037	-0.0025	0.0023	-0.0008	0.0000

Appendix 2 The metabolic profile of 57 identified compounds in GABA metabolic pathway mutants by GC-MS analysis.

Strain	Compound	2-Hydroxypyridine	3-Phosphoglyceric acid	5-Aminolevulinic acid	5-Methylthioadenosine	Adenine	2-Oxoglutaric acid	Alanine	Allothreonine	Asparagine	Aspartic acid	Citric acid	Citrulline	Cystathionine	Cysteine	Erythritol	Ethanolamine (2-aminoethanol)	Fumaric acid	γ-Aminobutyric acid	Glucose	Glutamic acid	Glutamine	Glyceric acid	Glycerol
WT-1		0.0291	0.0187	0.0317	0.0096	0.0148	0.0247	0.0297	0.0183	0.0274	0.0262	0.0253	0.0279	0.0366	0.0368	0.0367	0.0355	0.0440	0.0271	0.0400	0.0304	0.0265	0.0341	0.0389
WT-2		0.0260	0.0257	0.0370	0.0289	0.0280	0.0252	0.0253	0.0172	0.0256	0.0302	0.0193	0.0310	0.0269	0.0293	0.0283	0.0343	0.0365	0.0198	0.0297	0.0196	0.0262	0.0166	0.0239
WT-3		0.0349	0.0483	0.0354	0.0117	0.0015	0.0290	0.0418	0.0220	0.0282	0.0346	0.0152	0.0341	0.0319	0.0378	0.0006	0.0493	0.0272	0.0189	0.0444	0.0440	0.0352	0.0229	0.0320
WT-4		0.0470	0.0367	0.0262	0.0076	0.0268	0.0170	0.0266	0.0157	0.0208	0.0286	0.0187	0.0265	0.0304	0.0259	0.0206	0.0331	0.0241	0.0028	0.0393	0.0407	0.0279	0.0199	0.0285
WT-5		0.0501	0.0536	0.0187	0.0237	0.0206	0.0263	0.0327	0.0247	0.0255	0.0379	0.0187	0.0366	0.0358	0.0258	0.0111	0.0445	0.0424	0.0089	0.0700	0.0252	0.0337	0.0265	0.0366
uga1-1		0.0087	0.0091	0.0422	0.0264	0.0077	0.0195	0.0406	0.0982	0.0285	0.0282	0.0731	0.0297	0.0448	0.0276	0.0258	0.0288	0.0880	0.0277	0.0148	0.0261	0.0316	0.1061	0.0258
uga1-2		0.0126	0.0208	0.0735	0.0129	0.0212	0.0406	0.0210	0.0324	0.0332	0.0212	0.0476	0.0173	0.0283	0.0356	0.0257	0.0223	0.0421	0.0186	0.0102	0.0262	0.0221	0.0173	0.0185
uga1-3		0.0121	0.0258	0.0514	0.0320	0.0116	0.0181	0.0238	0.0340	0.0280	0.0257	0.0479	0.0190	0.0218	0.0191	0.0323	0.0188	0.0463	0.0021	0.0155	0.0163	0.0213	0.0258	0.0178
uga1-4		0.0214	0.0314	0.0504	0.0349	0.0203	0.0346	0.0404	0.0524	0.0305	0.0311	0.0526	0.0359	0.0357	0.0252	0.0123	0.0262	0.0535	0.0024	0.0314	0.0322	0.0295	0.0140	0.0205
uga1-5		0.0199	0.0306	0.0410	0.0074	0.0281	0.0210	0.0274	0.0335	0.0260	0.0234	0.0446	0.0206	0.0288	0.0297	0.0262	0.0211	0.0505	0.0097	0.0344	0.0323	0.0271	0.0401	0.0199
uga2-1		0.0112	0.0124	0.0001	0.0176	0.0185	0.0357	0.0312	0.0306	0.0400	0.0333	0.0215	0.0281	0.0256	0.0335	0.0311	0.0202	0.0146	0.1162	0.0122	0.0248	0.0270	0.0352	0.0315
uga2-2		0.0194	0.0023	0.0025	0.0005	0.0342	0.0279	0.0334	0.0429	0.0350	0.0338	0.0278	0.0355	0.0248	0.0309	0.0423	0.0262	0.0110	0.1053	0.0293	0.0304	0.0355	0.0190	0.0357
uga2-3		0.0283	0.0165	0.0004	0.0210	0.0212	0.0256	0.0281	0.0330	0.0361	0.0271	0.0096	0.0313	0.0251	0.0194	0.0308	0.0223	0.0102	0.1124	0.0122	0.0250	0.0298	0.0261	0.0341
uga2-4		0.0215	0.0266	0.0004	0.0004	0.0150	0.0283	0.0234	0.0222	0.0324	0.0262	0.0170	0.0359	0.0190	0.0251	0.0245	0.0238	0.0124	0.1550	0.0210	0.0262	0.0235	0.0338	0.0292
uga2-5		0.0296	0.0402	0.0005	0.0240	0.0240	0.0309	0.0340	0.0374	0.0471	0.0350	0.0229	0.0365	0.0272	0.0283	0.0248	0.0236	0.0088	0.1551	0.0124	0.0450	0.0357	0.0412	0.0352
uga3-1		0.0137	0.0097	0.0293	0.0643	0.0503	0.0273	0.0255	0.0185	0.0302	0.0279	0.0333	0.0255	0.0206	0.0279	0.0422	0.0231	0.0225	0.0004	0.0445	0.0203	0.0293	0.0166	0.0290
uga3-2		0.0199	0.0270	0.0273	0.0882	0.0620	0.0415	0.0297	0.0193	0.0281	0.0344	0.0424	0.0325	0.0202	0.0404	0.0520	0.0205	0.0269	0.0194	0.0397	0.0238	0.0366	0.0333	0.0391
uga3-3		0.0367	0.0234	0.0267	0.0534	0.0465	0.0133	0.0248	0.0156	0.0303	0.0293	0.0334	0.0292	0.0164	0.0241	0.0493	0.0211	0.0146	0.0005	0.0471	0.0246	0.0285	0.0211	0.0390
uga3-4		0.0278	0.0194	0.0231	0.0702	0.0447	0.0158	0.0220	0.0193	0.0245	0.0259	0.0269	0.0303	0.0198	0.0296	0.0510	0.0156	0.0171	0.0005	0.0265	0.0282	0.0336	0.0227	0.0321
uga3-5		0.0321	0.0305	0.0267	0.0395	0.0551	0.0307	0.0196	0.0205	0.0260	0.0225	0.0327	0.0268	0.0194	0.0221	0.0415	0.0182	0.0136	0.0064	0.0208	0.0276	0.0278	0.0231	0.0362
uga4-1		0.0260	0.0190	0.0250	0.0383	0.0137	0.0412	0.0325	0.0230	0.0288	0.0317	0.0175	0.0225	0.0255	0.0322	0.0329	0.0317	0.0455	0.0138	0.0327	0.0246	0.0273	0.0353	0.0277
uga4-2		0.0217	0.0226	0.0353	0.0139	0.0209	0.0197	0.0210	0.0125	0.0167	0.0240	0.0117	0.0185	0.0220	0.0208	0.0249	0.0206	0.0355	0.0196	0.0314	0.0151	0.0213	0.0282	0.0164
uga4-3		0.0221	0.0200	0.0271	0.0323	0.0157	0.0147	0.0235	0.0120	0.0201	0.0216	0.0125	0.0256	0.0249	0.0396	0.0166	0.0272	0.0317	0.0272	0.0288	0.0273	0.0246	0.0275	0.0186
uga4-4		0.0317	0.0279	0.0268	0.0203	0.0303	0.0220	0.0220	0.0151	0.0228	0.0254	0.0139	0.0255	0.0238	0.0332	0.0378	0.0302	0.0271	0.0141	0.0283	0.0263	0.0236	0.0342	0.0243
uga4-5		0.0543	0.0487	0.0348	0.0336	0.0203	0.0211	0.0328	0.0314	0.0325	0.0353	0.0126	0.0298	0.0281	0.0289	0.0007	0.0390	0.0344	0.0161	0.0203	0.0359	0.0355	0.0311	0.0471
gad1-1		0.0328	0.0278	0.0420	0.0420	0.0331	0.0577	0.0299	0.0248	0.0332	0.0301	0.0114	0.0282	0.0324	0.0407	0.0320	0.0282	0.0484	0.0122	0.0370	0.0291	0.0284	0.0341	0.0248
gad1-2		0.0310	0.0263	0.0224	0.0004	0.0343	0.0278	0.0256	0.0340	0.0273	0.0396	0.0156	0.0297	0.0310	0.0436	0.0374	0.0297	0.0398	0.0199	0.0108	0.0325	0.0325	0.0270	0.0260
gad1-3		0.0458	0.0605	0.0502	0.0367	0.0240	0.0331	0.0370	0.0307	0.0364	0.0259	0.0107	0.0251	0.0303	0.0202	0.0261	0.0388	0.0376	0.0116	0.0192	0.0393	0.0333	0.0251	0.0306
gad1-4		0.0313	0.0360	0.0300	0.0291	0.0347	0.0255	0.0242	0.0208	0.0290	0.0236	0.0125	0.0280	0.0286	0.0356	0.0294	0.0312	0.0271	0.0213	0.0449	0.0288	0.0285	0.0207	0.0273
gad1-5		0.0249	0.0299	0.0212	0.0014	0.0172	0.0339	0.0210	0.0178	0.0250	0.0224	0.0104	0.0246	0.0291	0.0281	0.0252	0.0256	0.0246	0.0176	0.0423	0.0271	0.0246	0.0266	0.0259
uga1gad1-1		0.0312	0.0317	0.0323	0.0302	0.0386	0.0258	0.0365	0.0420	0.0241	0.0345	0.0536	0.0241	0.0409	0.0184	0.0327	0.0404	0.0099	0.0083	0.0252	0.0267	0.0249	0.0192	0.0282
uga1gad1-2		0.0343	0.0317	0.0235	0.0468	0.0477	0.0381	0.0257	0.0222	0.0201	0.0252	0.0401	0.0285	0.0293	0.0162	0.0247	0.0284	0.0082	0.0021	0.0093	0.0255	0.0232	0.0255	0.0223
uga1gad1-3		0.0393	0.0344	0.0341	0.0418	0.0574	0.0259	0.0321	0.0407	0.0300	0.0304	0.0521	0.0336	0.0433	0.0287	0.0225	0.0380	0.0061	0.0039	0.0197	0.0299	0.0304	0.0229	0.0276
uga1gad1-4		0.0392	0.0353	0.0205	0.0388	0.0211	0.0431	0.0284	0.0390	0.0308	0.0263	0.0532	0.0378	0.0442	0.0225	0.0268	0.0361	0.0114	0.0005	0.0203	0.0342	0.0296	0.0251	0.0322
uga1gad1-5		0.0324	0.0397	0.0301	0.0203	0.0390	0.0375	0.0268	0.0261	0.0200	0.0214	0.0420	0.0281	0.0274	0.0172	0.0213	0.0261	0.0068	0.0025	0.0345	0.0288	0.0242	0.0219	0.0173

Appendix 2 Continued.

Strain	Compound	Glycine	Glycolic acid	Histidine	Homoserine	Hypoxanthine	Inositol	Isoleucine	Lactic acid	Lauric acid	Leucic acid	Leucine	Lysine	Malic acid	Mannose	Methionine	Nicotinic acid	Norleucine	Ornithine	Orotic acid	Phenylalanine	Phosphoric acid	Proline	Pyroglutamic acid
WT-1		0.0314	0.0219	0.0225	0.0286	0.0165	0.0170	0.0287	0.0299	0.0339	0.0260	0.0311	0.0489	0.0371	0.0247	0.0308	0.0167	0.0205	0.0334	0.0304	0.0379	0.0175	0.0328	0.0299
WT-2		0.0254	0.0293	0.0171	0.0164	0.0261	0.0131	0.0255	0.0219	0.0183	0.0265	0.0299	0.0192	0.0313	0.0323	0.0276	0.0175	0.0294	0.0262	0.0165	0.0233	0.0286	0.0237	0.0253
WT-3		0.0293	0.0097	0.0240	0.0339	0.0359	0.0181	0.0301	0.0274	0.0392	0.0380	0.0306	0.0290	0.0335	0.0376	0.0314	0.0238	0.0298	0.0320	0.0398	0.0247	0.0200	0.0391	0.0308
WT-4		0.0210	0.0137	0.0222	0.0251	0.0259	0.0150	0.0257	0.0309	0.0239	0.0069	0.0259	0.0308	0.0321	0.0252	0.0187	0.0203	0.0219	0.0211	0.0149	0.0214	0.0176	0.0273	0.0308
WT-5		0.0319	0.0198	0.0170	0.0348	0.0329	0.0161	0.0295	0.0379	0.0304	0.0172	0.0322	0.0201	0.0379	0.0320	0.0304	0.0172	0.0351	0.0340	0.0179	0.0250	0.0190	0.0343	0.0367
uga1-1		0.0349	0.0616	0.0321	0.0469	0.0661	0.1302	0.0454	0.0484	0.0417	0.0522	0.0328	0.0233	0.0582	0.0459	0.0344	0.0554	0.0201	0.0263	0.0288	0.0189	0.0495	0.0334	0.0362
uga1-2		0.0298	0.0183	0.0420	0.0350	0.0358	0.0917	0.0317	0.0241	0.0260	0.0431	0.0337	0.0179	0.0392	0.0584	0.0296	0.0258	0.0114	0.0204	0.0120	0.0223	0.0283	0.0274	0.0327
uga1-3		0.0319	0.0292	0.0335	0.0277	0.0295	0.0869	0.0298	0.0331	0.0280	0.0266	0.0291	0.0264	0.0310	0.0210	0.0261	0.0237	0.0159	0.0244	0.0143	0.0181	0.0269	0.0301	0.0271
uga1-4		0.0392	0.0250	0.0350	0.0369	0.0304	0.1337	0.0359	0.0331	0.0383	0.0388	0.0288	0.0300	0.0444	0.0392	0.0298	0.0212	0.0412	0.0236	0.0198	0.0179	0.0193	0.0297	0.0339
uga1-5		0.0281	0.0431	0.0388	0.0355	0.0187	0.0877	0.0295	0.0269	0.0354	0.0341	0.0258	0.0239	0.0426	0.0308	0.0292	0.0122	0.0348	0.0237	0.0148	0.0160	0.0316	0.0223	0.0296
uga2-1		0.0295	0.0514	0.0222	0.0376	0.0002	0.0197	0.0307	0.0264	0.0212	0.0641	0.0204	0.0301	0.0128	0.0326	0.0263	0.0631	0.0269	0.0361	0.0465	0.0362	0.0289	0.0269	0.0285
uga2-2		0.0403	0.0590	0.0473	0.0368	0.0110	0.0204	0.0387	0.0294	0.0416	0.0612	0.0091	0.0314	0.0200	0.0458	0.0319	0.0748	0.0420	0.0512	0.0633	0.0447	0.0600	0.0365	0.0344
uga2-3		0.0318	0.0257	0.0257	0.0446	0.0164	0.0161	0.0249	0.0303	0.0286	0.0384	0.0259	0.0196	0.0131	0.0321	0.0209	0.0523	0.0324	0.0372	0.0415	0.0320	0.0209	0.0237	0.0281
uga2-4		0.0291	0.0341	0.0287	0.0226	0.0183	0.0131	0.0291	0.0294	0.0339	0.0407	0.0246	0.0297	0.0129	0.0337	0.0252	0.0407	0.0182	0.0432	0.0418	0.0268	0.0370	0.0307	0.0340
uga2-5		0.0403	0.0512	0.0574	0.0302	0.0364	0.0259	0.0300	0.0359	0.0299	0.0361	0.0348	0.0239	0.0175	0.0178	0.0284	0.0597	0.0324	0.0493	0.0354	0.0476	0.0345	0.0373	0.0431
uga3-1		0.0192	0.0443	0.0139	0.0249	0.0010	0.0103	0.0234	0.0357	0.0276	0.0299	0.0233	0.0455	0.0275	0.0270	0.0265	0.0237	0.0223	0.0283	0.0076	0.0309	0.0200	0.0265	0.0201
uga3-2		0.0257	0.0380	0.0386	0.0328	0.0010	0.0142	0.0280	0.0311	0.0227	0.0130	0.0376	0.0118	0.0281	0.0178	0.0397	0.0179	0.0385	0.0363	0.0127	0.0448	0.0340	0.0279	0.0233
uga3-3		0.0203	0.0316	0.0212	0.0266	0.0362	0.0087	0.0221	0.0331	0.0288	0.0016	0.0257	0.0091	0.0218	0.0207	0.0335	0.0135	0.0561	0.0336	0.0070	0.0367	0.0169	0.0200	0.0229
uga3-4		0.0237	0.0329	0.0385	0.0271	0.0304	0.0115	0.0217	0.0313	0.0209	0.0137	0.0278	0.0530	0.0206	0.0146	0.0270	0.0184	0.0393	0.0285	0.0012	0.0341	0.0348	0.0202	0.0297
uga3-5		0.0198	0.0244	0.0422	0.0242	0.0583	0.0085	0.0233	0.0253	0.0247	0.0223	0.0318	0.0343	0.0176	0.0197	0.0315	0.0268	0.0234	0.0303	0.0114	0.0387	0.0320	0.0261	0.0244
uga4-1		0.0289	0.0240	0.0149	0.0174	0.0349	0.0212	0.0278	0.0346	0.0367	0.0559	0.0313	0.0285	0.0430	0.0239	0.0315	0.0261	0.0364	0.0295	0.0375	0.0315	0.0328	0.0294	0.0261
uga4-2		0.0213	0.0138	0.0135	0.0144	0.0359	0.0091	0.0247	0.0230	0.0296	0.0326	0.0212	0.0259	0.0314	0.0201	0.0200	0.0241	0.0135	0.0185	0.0332	0.0164	0.0277	0.0277	0.0215
uga4-3		0.0303	0.0255	0.0217	0.0111	0.0408	0.0136	0.0245	0.0130	0.0319	0.0346	0.0267	0.0252	0.0375	0.0153	0.0249	0.0221	0.0199	0.0211	0.0250	0.0182	0.0189	0.0281	0.0238
uga4-4		0.0290	0.0176	0.0267	0.0148	0.0415	0.0251	0.0214	0.0119	0.0249	0.0223	0.0225	0.0330	0.0345	0.0225	0.0227	0.0294	0.0329	0.0243	0.0317	0.0199	0.0326	0.0233	0.0253
uga4-5		0.0365	0.0359	0.0368	0.0435	0.0378	0.0165	0.0330	0.0256	0.0424	0.0353	0.0329	0.0332	0.0335	0.0155	0.0397	0.0511	0.0168	0.0270	0.0305	0.0303	0.0426	0.0350	0.0316
gad1-1		0.0247	0.0186	0.0184	0.0238	0.0009	0.0189	0.0301	0.0228	0.0273	0.0287	0.0371	0.0533	0.0387	0.0136	0.0327	0.0226	0.0423	0.0252	0.0323	0.0307	0.0300	0.0296	0.0324
gad1-2		0.0280	0.0199	0.0411	0.0260	0.0297	0.0197	0.0319	0.0172	0.0290	0.0282	0.0318	0.0320	0.0443	0.0213	0.0312	0.0257	0.0272	0.0222	0.0461	0.0313	0.0235	0.0322	0.0323
gad1-3		0.0326	0.0389	0.0349	0.0288	0.0385	0.0167	0.0331	0.0136	0.0275	0.0334	0.0390	0.0418	0.0329	0.0247	0.0344	0.0285	0.0412	0.0311	0.0404	0.0353	0.0290	0.0349	0.0345
gad1-4		0.0255	0.0262	0.0254	0.0208	0.0525	0.0250	0.0249	0.0134	0.0224	0.0359	0.0279	0.0216	0.0378	0.0176	0.0293	0.0353	0.0290	0.0237	0.0424	0.0295	0.0329	0.0287	0.0278
gad1-5		0.0261	0.0270	0.0219	0.0160	0.0231	0.0140	0.0231	0.0149	0.0312	0.0162	0.0270	0.0231	0.0301	0.0191	0.0256	0.0365	0.0194	0.0260	0.0499	0.0358	0.0307	0.0231	0.0279
uga1gad1-1		0.0263	0.0111	0.0273	0.0390	0.0330	0.0132	0.0322	0.0556	0.0308	0.0016	0.0312	0.0271	0.0088	0.0455	0.0280	0.0158	0.0189	0.0197	0.0292	0.0254	0.0200	0.0324	0.0189
uga1gad1-2		0.0234	0.0170	0.0253	0.0271	0.0230	0.0100	0.0264	0.0343	0.0137	0.0151	0.0258	0.0257	0.0108	0.0235	0.0240	0.0137	0.0269	0.0177	0.0274	0.0208	0.0124	0.0206	0.0206
uga1gad1-3		0.0306	0.0136	0.0375	0.0350	0.0406	0.0156	0.0346	0.0385	0.0187	0.0185	0.0380	0.0261	0.0143	0.0515	0.0290	0.0267	0.0248	0.0259	0.0329	0.0324	0.0278	0.0353	0.0258
uga1gad1-4		0.0291	0.0221	0.0118	0.0319	0.0172	0.0129	0.0289	0.0330	0.0185	0.0017	0.0211	0.0300	0.0131	0.0461	0.0259	0.0173	0.0246	0.0292	0.0298	0.0217	0.0329	0.0229	0.0282
uga1gad1-5		0.0253	0.0245	0.0228	0.0224	0.0237	0.0104	0.0196	0.0271	0.0202	0.0097	0.0254	0.0158	0.0102	0.0307	0.0221	0.0002	0.0343	0.0198	0.0341	0.0227	0.0291	0.0207	0.0219

Appendix 2 Continued.

Strain	Compound	Pyruvic acid	Ribose	Serine	Succinic acid	Threonine	Trehalose	Tryptophane	Tyrosine	Uracil	Urea	Valine
WT-1		0.0256	0.0162	0.0344	0.0447	0.0272	0.0362	0.0290	0.0253	0.0270	0.0350	0.0306
WT-2		0.0284	0.0228	0.0266	0.0324	0.0188	0.0130	0.0245	0.0221	0.0257	0.0495	0.0235
WT-3		0.0393	0.0368	0.0369	0.0253	0.0270	0.0120	0.0269	0.0228	0.0355	0.0521	0.0250
WT-4		0.0292	0.0280	0.0289	0.0308	0.0201	0.0314	0.0233	0.0189	0.0266	0.0486	0.0239
WT-5		0.0310	0.0184	0.0302	0.0368	0.0244	0.0002	0.0210	0.0222	0.0257	0.0674	0.0301
uga1-1		0.0365	0.0222	0.0315	0.0613	0.0391	0.0503	0.0305	0.0251	0.0383	0.0536	0.0352
uga1-2		0.0488	0.0406	0.0332	0.0330	0.0394	0.0488	0.0217	0.0201	0.0290	0.0335	0.0231
uga1-3		0.0348	0.0337	0.0251	0.0297	0.0319	0.0401	0.0238	0.0186	0.0275	0.0229	0.0182
uga1-4		0.0423	0.0239	0.0335	0.0465	0.0424	0.0403	0.0228	0.0214	0.0372	0.0460	0.0278
uga1-5		0.0326	0.0231	0.0279	0.0404	0.0321	0.0506	0.0291	0.0203	0.0324	0.0399	0.0181
uga2-1		0.0338	0.0246	0.0410	0.0036	0.0354	0.0415	0.0471	0.0361	0.0310	0.0515	0.0317
uga2-2		0.0332	0.0333	0.0375	0.0039	0.0433	0.0293	0.0572	0.0407	0.0381	0.0004	0.0367
uga2-3		0.0293	0.0278	0.0320	0.0026	0.0349	0.0327	0.0299	0.0314	0.0280	0.0460	0.0300
uga2-4		0.0283	0.0223	0.0302	0.0034	0.0291	0.0344	0.0333	0.0339	0.0279	0.0588	0.0256
uga2-5		0.0293	0.0347	0.0394	0.0039	0.0381	0.0373	0.0302	0.0360	0.0381	0.0581	0.0344
uga3-1		0.0253	0.0233	0.0212	0.0248	0.0162	0.0398	0.0318	0.0360	0.0235	0.0305	0.0310
uga3-2		0.0245	0.0219	0.0291	0.0283	0.0198	0.0461	0.0389	0.0474	0.0247	0.0004	0.0358
uga3-3		0.0189	0.0191	0.0247	0.0215	0.0205	0.0316	0.0276	0.0471	0.0289	0.0259	0.0323
uga3-4		0.0167	0.0207	0.0253	0.0222	0.0170	0.0441	0.0328	0.0454	0.0228	0.0004	0.0244
uga3-5		0.0194	0.0206	0.0256	0.0196	0.0160	0.0375	0.0339	0.0473	0.0238	0.0001	0.0262
uga4-1		0.0313	0.0363	0.0246	0.0368	0.0238	0.0176	0.0346	0.0223	0.0311	0.0442	0.0295
uga4-2		0.0224	0.0228	0.0177	0.0328	0.0174	0.0122	0.0229	0.0132	0.0210	0.0212	0.0191
uga4-3		0.0249	0.0316	0.0196	0.0282	0.0209	0.0102	0.0163	0.0202	0.0228	0.0314	0.0230
uga4-4		0.0269	0.0407	0.0197	0.0301	0.0194	0.0008	0.0263	0.0218	0.0242	0.0001	0.0187
uga4-5		0.0405	0.0605	0.0342	0.0333	0.0363	0.0201	0.0285	0.0242	0.0418	0.0004	0.0308
gad1-1		0.0296	0.0233	0.0308	0.0426	0.0257	0.0188	0.0278	0.0250	0.0272	0.0001	0.0333
gad1-2		0.0292	0.0191	0.0258	0.0388	0.0265	0.0197	0.0327	0.0237	0.0276	0.0006	0.0290
gad1-3		0.0332	0.0260	0.0328	0.0505	0.0267	0.0227	0.0231	0.0313	0.0369	0.0229	0.0324
gad1-4		0.0222	0.0240	0.0275	0.0383	0.0239	0.0152	0.0310	0.0288	0.0211	0.0001	0.0258
gad1-5		0.0214	0.0260	0.0227	0.0312	0.0231	0.0002	0.0249	0.0226	0.0216	0.0003	0.0203
uga1gad1-1		0.0231	0.0359	0.0318	0.0241	0.0366	0.0176	0.0250	0.0279	0.0315	0.0482	0.0416
uga1gad1-2		0.0221	0.0269	0.0209	0.0201	0.0307	0.0366	0.0207	0.0284	0.0240	0.0376	0.0323
uga1gad1-3		0.0240	0.0364	0.0308	0.0293	0.0468	0.0536	0.0256	0.0380	0.0276	0.0410	0.0425
uga1gad1-4		0.0229	0.0497	0.0268	0.0293	0.0368	0.0442	0.0268	0.0306	0.0287	0.0001	0.0334
uga1gad1-5		0.0191	0.0269	0.0201	0.0197	0.0325	0.0131	0.0183	0.0238	0.0212	0.0311	0.0244

Appendix 3 The metabolic profile of 37 compounds in senescent cells by GC-MS analysis

			1st						4th						7th						11th					
Compounds	RT	QuantMS	#1	#2	#3	#4	AV	SD	#1	#2	#3	#4	AV	SD	#1	#2	#3	#4	AV	SD	#1	#2	#3	#4	AV	SD
Amino acid																										
γ-Aminobutyric acid	570.50	174	0.4842	0.8311	0.9658	1.0365	0.8294	0.2125	2.0187	1.5233	1.2821	1.0897	1.4784	0.3477	1.1719	1.1765	1.3388	1.3310	1.2545	0.0804	0.7569	0.8965	0.9520	0.5822	0.7969	0.1429
Alanine	318.55	116	0.7956	0.4380	0.7886	0.4846	0.6267	0.1662	0.7463	1.5696	0.7435	0.8586	0.9795	0.3438	0.5476	0.7352	1.3419	1.6529	1.0694	0.4468	0.7992	1.1747	0.9521	0.7397	0.9164	0.1681
Arginine + Ornithine	700.35	142	2.0044	1.9021	1.7223	2.3276	1.9891	0.2200	1.5648	1.3813	1.3815	1.9483	1.5690	0.2314	2.0536	1.6943	1.4347	1.8292	1.7529	0.2241	1.6356	2.0976	1.7779	1.0202	1.6328	0.3913
Asparagine	635.25	116	0.0256	0.0191	0.1032	0.0694	0.0543	0.0342	0.1118	0.2258	0.1590	0.1257	0.1556	0.0440	0.0275	0.0618	0.2008	0.2822	0.1431	0.1033	0.0917	0.0960	0.0915	0.0720	0.0878	0.0093
Aspartic acid	561.75	232	0.7611	0.5141	0.6087	0.6064	0.6226	0.0886	1.3989	1.7603	1.2712	1.6397	1.5175	0.1928	1.1536	1.2957	1.5174	1.8102	1.4442	0.2479	1.2463	1.4074	1.3926	1.0768	1.2808	0.1335
Citruline	703.70	157	0.0381	0.0246	0.0328	0.0266	0.0305	0.0053	0.0402	0.0514	0.0304	0.0295	0.0379	0.0089	0.0469	0.0520	0.0555	0.0590	0.0534	0.0045	0.0465	0.0423	0.0508	0.0375	0.0443	0.0049
Glutamic acid	610.85	246	3.3608	2.8808	2.9849	3.0433	3.0674	0.1791	6.4507	6.4439	6.2522	7.3621	6.6272	0.4317	4.4452	4.8083	5.0849	5.6359	4.9936	0.4347	3.8457	3.6746	3.5759	2.8838	3.4950	0.3658
Glutamine	682.25	156	0.0731	0.0632	0.1114	0.0470	0.0737	0.0237	0.8484	0.9669	0.5802	0.3898	0.6963	0.2257	0.5744	0.6071	0.6750	0.6380	0.6236	0.0372	0.5272	0.1854	0.3706	0.3555	0.3597	0.1210
Glycine	447.85	174	1.8778	1.4321	1.9587	1.7309	1.7499	0.2008	2.1431	2.9116	1.6780	1.9468	2.1699	0.4589	1.2026	1.4456	2.2414	2.9173	1.9517	0.6771	1.2015	1.3612	1.4369	1.1207	1.2801	0.1252
Histidine	745.40	154	0.2703	0.2700	0.2407	0.2395	0.2551	0.0150	0.2734	0.3065	0.2136	0.2257	0.2548	0.0373	0.2057	0.2730	0.3079	0.3392	0.2815	0.0496	0.1644	0.2215	0.2078	0.1338	0.1819	0.0349
Homoserine	525.10	218	0.0338	0.0333	0.0248	0.0294	0.0303	0.0036	0.0601	0.0494	0.0324	0.0396	0.0454	0.0104	0.0191	0.0190	0.0206	0.0326	0.0228	0.0057	0.0357	0.0390	0.0367	0.0247	0.0340	0.0055
Isoleucine	439.20	158	0.3098	0.2201	0.5820	0.2545	0.3416	0.1425	0.6477	1.3774	0.6789	0.5732	0.8193	0.3245	0.2004	0.3480	1.0119	1.4545	0.7537	0.5071	0.3206	0.2362	0.3297	0.3966	0.3208	0.0570
Leucine	426.10	158	0.4572	0.3128	1.0353	0.4086	0.5535	0.2830	1.1368	2.4481	1.2693	1.0933	1.4869	0.5588	0.3364	0.5976	2.0595	2.8618	1.4638	1.0405	0.6712	0.4901	0.6925	0.7837	0.6594	0.1065
Lysine	744.15	156	2.4749	2.5275	2.5785	2.6847	2.5664	0.0775	3.1831	2.8488	2.4973	2.8432	2.8431	0.2425	3.8876	3.7211	4.0691	4.4192	4.0243	0.2591	4.2991	5.0180	4.7440	3.0717	4.2832	0.7450
Methionine	564.80	176	0.0432	0.0268	0.0824	0.0353	0.0469	0.0213	0.1405	0.2519	0.1628	0.1494	0.1762	0.0444	0.0580	0.1011	0.2202	0.2829	0.1655	0.0901	0.0709	0.0650	0.0888	0.0788	0.0759	0.0089
Phenylalanine	618.90	218	0.1601	0.1049	0.3331	0.1304	0.1821	0.0893	0.3936	0.7858	0.4252	0.3294	0.4835	0.1779	0.1245	0.2254	0.7300	0.9702	0.5125	0.3499	0.2497	0.2127	0.2875	0.2911	0.2603	0.0319
Proline	444.25	142	0.3566	0.2266	0.3517	0.2482	0.2958	0.0589	0.5932	1.0222	0.6870	0.8761	0.7946	0.1663	0.4048	0.5119	0.6841	0.8500	0.6127	0.1694	0.5591	0.6748	0.6192	0.5714	0.6061	0.0455
Pyroglutamic acid	567.65	156	1.2864	1.2426	1.8935	1.4502	1.4682	0.2574	2.4852	3.4851	2.4383	2.1802	2.6472	0.4975	2.2441	2.7404	3.9242	4.7252	3.4085	0.9749	2.4346	2.0819	2.6200	2.2924	2.3572	0.1969
Serine	475.85	204	0.3455	0.1557	0.2739	0.2054	0.2451	0.0715	0.4186	0.7663	0.3992	0.5110	0.5238	0.1463	0.2310	0.3269	0.6119	0.8279	0.4994	0.2358	0.3907	0.5349	0.5431	0.4205	0.4723	0.0676
Threonine	490.05	117	0.0958	0.0634	0.1032	0.0804	0.0857	0.0153	0.1915	0.2929	0.1530	0.1636	0.2002	0.0553	0.1134	0.1549	0.2349	0.2817	0.1962	0.0659	0.1320	0.1499	0.1655	0.1359	0.1458	0.0132
Tryptophan	860.65	202	0.0222	0.0112	0.0791	0.0242	0.0342	0.0264	0.0996	0.2299	0.0897	0.0630	0.1206	0.0645	0.0194	0.0373	0.1698	0.2758	0.1256	0.1044	0.0661	0.0593	0.0713	0.0664	0.0657	0.0043
Tyrosine	752.65	218	0.2367	0.2179	0.5025	0.2580	0.3038	0.1156	0.5639	0.9991	0.5325	0.4324	0.6320	0.2175	0.2253	0.3308	0.9285	1.2657	0.6876	0.4281	0.3310	0.2817	0.3764	0.3294	0.3296	0.0335
Valine	392.00	144	0.4130	0.3515	0.6355	0.3602	0.4401	0.1153	0.8787	1.1692	0.6368	0.5787	0.8158	0.2330	0.3796	0.4757	0.8269	1.2173	0.7249	0.3295	0.2843	0.1970	0.3049	0.3080	0.2735	0.0451
Organic acid																										
2-Oxoglutaric acid	589.00	89	0.0181	0.0111	0.0141	0.0155	0.0147	0.0025	0.0101	0.0124	0.0145	0.0137	0.0127	0.0017	0.0162	0.0141	0.0140	0.0124	0.0142	0.0013	0.0113	0.0105	0.0188	0.0170	0.0144	0.0036
Citric acid + Isocitric acid	698.80	273	0.0565	0.0475	0.0925	0.0774	0.0685	0.0176	0.5159	0.5551	0.5055	0.6104	0.5467	0.0412	0.2493	0.3746	0.4289	0.4562	0.3772	0.0795	0.7895	1.0055	1.0504	0.6760	0.8804	0.1538
Fumaric acid	472.55	245	0.0089	0.0074	0.0071	0.0051	0.0071	0.0013	0.0115	0.0133	0.0055	0.0055	0.0089	0.0035	0.0113	0.0103	0.0102	0.0151	0.0117	0.0020	0.0101	0.0110	0.0100	0.0114	0.0106	0.0006
Lauric acid	630.05	117	0.0173	0.0126	0.0261	0.0136	0.0174	0.0053	0.0267	0.0307	0.0222	0.0164	0.0240	0.0053	0.0178	0.0380	0.0192	0.0169	0.0230	0.0087	0.0189	0.0143	0.0241	0.0168	0.0185	0.0036
Maleic acid	452.00	147	0.1701	0.1642	0.3996	0.1805	0.2286	0.0989	0.3672	0.6640	0.3811	0.2911	0.4259	0.1417	0.1008	0.1523	0.5181	0.7644	0.3839	0.2723	0.2509	0.1940	0.3012	0.2364	0.2456	0.0383
Malic acid	539.10	133	0.0977	0.0804	0.0861	0.0830	0.0868	0.0066	0.0766	0.0793	0.1003	0.0946	0.0877	0.0100	0.1250	0.1109	0.1009	0.1144	0.1128	0.0086	0.1136	0.0823	0.1150	0.0878	0.0997	0.0148
Pyruvic acid + Oxalacetic acid	283.10	174	0.0276	0.0065	0.0068	0.0107	0.0129	0.0086	0.0094	0.0119	0.0116	0.0132	0.0115	0.0014	0.0107	0.0170	0.0325	0.0253	0.0213	0.0083	0.0253	0.0273	0.0284	0.0369	0.0295	0.0044
Shikimic acid	691.60	204	0.0050	0.0063	0.0098	0.0079	0.0073	0.0018	0.0135	0.0247	0.0205	0.0120	0.0177	0.0052	0.0063	0.0110	0.0283	0.0351	0.0202	0.0119	0.0190	0.0238	0.0261	0.0184	0.0218	0.0032
Succinic acid (or aldehyde)	451.85	247	0.0197	0.0231	0.0458	0.0243	0.0282	0.0103	0.0492	0.0803	0.0470	0.0375	0.0535	0.0161	0.0101	0.0199	0.0643	0.0906	0.0462	0.0327	0.0294	0.0242	0.0357	0.0306	0.0300	0.0041
Sugar/Alcohol																										
Glucose	730.20	319	0.5716	0.4828	12.1338	1.6300	3.7045	4.8875	16.0911	23.5505	19.7836	12.9829	18.1020	3.9611	0.9353	4.3921	24.2616	24.0051	13.3985	10.8045	7.2834	5.5379	10.6107	9.5034	8.2338	1.9642
Glycerol	424.90	147	2.7591	2.2384	3.8349	2.4641	2.8241	0.6121	13.3460	14.2300	11.6378	12.4126	12.9066	0.9745	11.0148	13.8454	12.6319	12.7019	12.5485	1.0080	10.0657	11.4036	12.4354	8.2575	10.5406	1.5631
Internal standard																										
Ribitol	655.75	217	1.0000	1.0000	1.0000	1.0000			1.0000	1.0000	1.0000	1.0000			1.0000	1.0000	1.0000	1.0000			1.0000	1.0000	1.0000	1.0000		