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Gene Expression Profiles of Yeast *Saccharomyces cerevisiae sod1* Caused by Patulin Toxicity and Evaluation of Recovery Potential of Ascorbic Acid

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ABSTRACT: Patulin (PAT) is a fungal secondary metabolite and exhibits various toxicities including DNA damage and oxidative stress. These toxicities are eased by ascorbic acid (AsA). Although a number of studies regarding the mitigating effect of AsA against PAT toxicity have been reported, a comprehensive study about gene expressions is currently underway. Here, we carried out a detailed evaluation of PAT toxicity by co-incubation with AsA using the superoxide dismutase (SOD) mutant. DNA microarray results extracted the alterations in iron transporter and Fe/S cluster assembly genes; some of the genes that constitute the cellular iron transporter systems remained dysfunctional even in the presence of AsA. Meanwhile, AsA treatment reduced the alterations of G1/S phase cell cycle regulation genes. These results suggest that oxidative stress-derived DNA damage still exists, although AsA treatment effectively reduces PAT toxicity. This implies that a combined condition is required for complete blockade of PAT toxicity.

KEYWORDS: SOD, yeast, DNA microarray, ascorbic acid, patulin, Fe/S cluster

INTRODUCTION

Patulin (PAT), a secondary metabolite generated by several fungi, including *Penicillium* and *Aspergillus*, is known to be a harmful substance. Various fruits, including apple, can be contaminated by fungi and pose a toxic risk to the drinks and foods derived from them. PAT toxicity causes edema and hemorrhage and is suspected to be involved in carcinogenicity according to a number of previous publications.[1−3] Cell-based studies indicate that PAT causes G1/S arrest in murine skin cells and also G2/M arrest in V79 Chinese hamster cells.[4,5] Thus, the effect of PAT on cell cycle arrest seems to be dependent on the subject of research. Chromosome-type and chromatid-type gaps and breaks were also induced by PAT in Chinese hamster ovary cells, but not in human peripheral blood lymphocytes.[6] DNA damage, such as the inhibition of DNA repair and replication, is well reported, and mutagenicity has been indicated in several studies, although it is reported that PAT does not pose a risk for carcinogenicity.[7−9] The possibility of carcinogenicity remains inconclusive; therefore, continued accumulation of extensively detailed data is required.

PAT interacts with plasma membrane proteins and couples to the sulfhydryl group of glutathione or cysteine.[10] This reaction inhibits the reducing activity on membrane proteins and causes dysfunction in Na^+/K^+ ion transport.[11] These changes cause a decrease in stress resistance against exogenous stressors and also cause a decrease in the scavenging ability for oxygen radicals. This induces an increase in oxygen radicals and causes DNA damage. Research using yeast (*Saccharomyces cerevisiae* strain S288C) supports these mechanisms of toxicity and has also indicated the involvement of a significant oxidative stress response and DNA damage genes.[12]

Various antioxidative mechanisms function to protect cells against oxidative stress and, in particular, superoxide dismutase (SOD), which encodes superoxide dismutase and functions in the redox reaction, is important to the antioxidative response. Suppression of SOD elevates the susceptibility to PAT toxicity.[13] Additionally, our previous study reported that the *SOD1* disruptant (*sod1* mutant, a BY4743 derivative strain) exhibited very high susceptibility to PAT in the presence of sodium dodecyl sulfate.[14] It is possible to measure cellular oxidation using the *sod1* mutant. Tamura[15] recently reported that the addition of 10 mM ascorbic acid (AsA) abrogates sorbitol-induced oxidative stress. With respect to the relationship between AsA and PAT, several studies investigating PAT toxicity reduction have been reported.[16−18] The addition of 80 μM AsA to V79 cells diminished the risk of PAT-induced clastogenicity, although the genotoxic risk was not eliminated completely.[17] It is also suggested that the simple provision of an ascorbic acid condition is not appropriate for reducing PAT toxicity in rumen or bacterial studies.[16,18] One of the reasons for continued risk is because AsA produces antioxidative activity and also plays a role as a prooxidant with redox-active transition metal ions.[19−21] SOD also generates hydrogen peroxide from radical oxygen, and oxidative stress is induced by the coexistence of hydrogen peroxide, AsA, and redox-active transition metal ion.[22] However, AsA ordinarily exerts antioxidative stress reaction effects rather than a prooxidant reaction;[23] therefore, it is thought that this contributes to the mitigation of oxidative DNA stress by PAT. In another study using smooth muscle cells, SOD is induced by sufficient doses of AsA.[24] In addition to those papers, to eliminate PAT toxicity completely, a comprehensive genetic study is required because

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there is little information about the behavior of genes as a result of the relationship between AsA and PAT. Fortunately, our group had analyzed PAT toxicity using DNA microarray and also reported that the *sod1* mutant is highly susceptible to PAT. From the combination of those studies, it was expected that the effect of AsA against PAT could be more clearly indicated. Here, we determined gene expression changes in the *sod1* mutant with AsA and PAT treatment to investigate PAT toxicity in more detail and identified gene categories that represent potential mechanisms for recovery from PAT toxicity.

MATERIALS AND METHODS

Yeast Strain and Chemicals. The yeast (*S. cerevisiae*) strain BY4743 derivative, *sod1* mutant, which lacks the superoxide dismutase coding gene (*SOD1*) and was obtained from Thermo Fisher Scientific (Waltham, MA), was used in this study. The mycotoxin patulin (Wako, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO), and L-(+)-ascorbic acid (Wako) was dissolved in distilled water.

Growth Test. Strain *sod1* was preincubated until reaching the stationary phase (A_{650} = 1.0) and diluted to 20-fold with YPD medium (1% yeast extract, 2% polypeptone, and 2% glucose). The diluted culture solution (200 μL) was dispensed into the wells of a 96-well flat-bottom microplate, and DMSO, AsA (final 10 mM) or PAT (final 25 ppm), or both AsA and PAT were added, and the culture plate was incubated at 25 °C on an automatic pipetting device (Biomek2000; Beckman Coulter, Brea, CA); each well was agitated by pipetting every 2 h. A growth curve for each condition was determined by reading the absorbance (OD_{650}) of at least three replicates.

RNA Preparation and DNA Microarray. For RNA purification, yeast cultures (A_{650} = 1.0, 10 mL) were grown at 25 °C in YPD media with 10 mM AsA or 25 ppm PAT or both AsA and PAT, respectively. The same volume of DMSO was added into the culture medium as a control. Culture solutions were incubated with 150 rpm shaking at 25 °C for 2 h. Cell pellets were obtained by centrifugation at 3000 rpm for 5 min, and total RNA was extracted using a commercial kit (FastRNA Pro Red kit, MP Biomedicals, Irvine, CA) following the manufacturer's instructions. To avoid contamination of genomic DNA, samples were DNase-treated before reverse transcription. DNA microarrays (GeneChip Yeast Genome 2.0 array, Affymetrix, Santa Clara, CA) were processed according to the manufacturer's instructions. Array data were transferred into GeneSpring analysis software (ver. 9, Agilent Technologies, Santa Clara, CA), and cluster analyses were performed for each condition. After the MAS5 algorithm had been used to obtain summarized probeset-level expression data, the average expression of three replicates was normalized to the control condition. An unpaired *t* test was used for statistical analysis, and significant differences in gene expression were selected using a *p* value of <0.05. To avoid the detection of false positives, a multiple testing correction (Benjamini-Hochberg FDR) was applied to obtain corrected *p* values. Selected genes were categorized according to the Munich Information Center for Protein Sequences (MIPS, http://mips.gsf.de/) and Saccharomyces Genome Database (SGD, http://www.yeastgenome.org/) annotation. The microarray data set has been assigned the accession number GSE26171 in the Gene Expression Omnibus Database (GEO, http://www.ncbi.nlm.nih.gov/geo/).

RESULTS AND DISCUSSION

AsA Recovers *sod1* Mutant Growth in the Presence of PAT. PAT binds the sulfhydryl groups of membrane proteins and causes a reduction in glutathione activity.[25] Glutathione plays an important role in maintaining stable intracellular conditions.[8] With the loss of that function, oxygen radical species easily damage cellular components including nucleotides. We employed the *S. cerevisiae sod1* mutant, which lacks redox ability, to examine the role of oxidative stress in PAT pathology. Our previous paper showed that the *sod1* mutant was more susceptible to PAT than a number of other mutant strains.[14] In the present study, we investigated the relationship between PAT concentration and growth inhibition in the *sod1* mutant. The results indicated that inhibition of *sod1* mutant growth was proportional to the concentration of PAT, and 25 ppm PAT significantly inhibited growth (Figure 1a). Iwahashi[12] examined PAT toxicity in the S288C yeast strain, using a higher concentration than in the present study. Therefore, it is thought that the *sod1* mutant is a more sensitive yeast strain than S288C if the same outcome is expected.

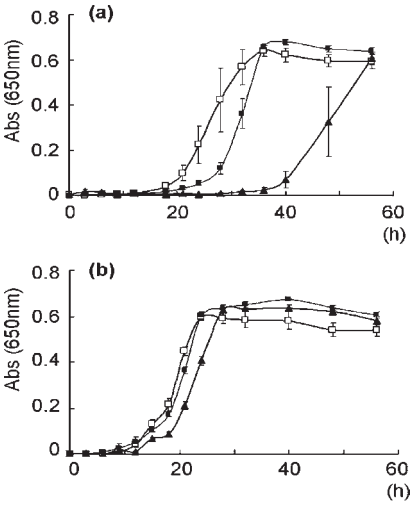

Figure 1. Growth delay caused by PAT treatment and the restorative effect of AsA: (a) cells were incubated under normal conditions; (b) cells were co-incubated with 10 mM AsA. Yeast *sod1* cells were treated with 10 ppm PAT (gray squares), 25 ppm PAT (triangles), and DMSO as a control (open squares). Individual data were obtained from three replicates. Bars = standard error.

Tamura[15] reported that the addition of 10 mM AsA protects the *sod1* mutant from oxidative stress. In reference to that study, we utilized the same AsA concentration and attempted to counteract PAT toxicity. As a result, the growth of the PAT-exposed *sod1* mutant was greatly improved by AsA treatment, although it seemed that the inhibitory influence of PAT remained (Figure 1). In the absence of AsA, the growth suppression of the *sod1* mutant was proportional to the concentration of PAT, and 25 ppm PAT exposure significantly inhibited growth (Figure 1a). Conversely, *sod1* mutant with AsA treatment exhibited only 3 h of growth delay at most by PAT exposure (Figure 1b). These results demonstrated that the addition of AsA is effective in relieving PAT toxicity, even though it was not possible to evaluate whether the toxicological risk was eliminated completely. To address this question, we then observed gene expression changes to obtain further knowledge of the relationship between AsA and PAT toxicity. A total of four series of samples were used in the DNA microarray analysis: control, 10 mM AsA, 25 ppm PAT, and AsA + PAT treatments. A total of 813 genes were identified as altered using the following inclusion criteria: >1.5- or <0.67-fold change with subsequent statistical analysis using ANOVA. In the present study, we extracted the genes that exhibited a >1.5-fold difference in any comparison of the

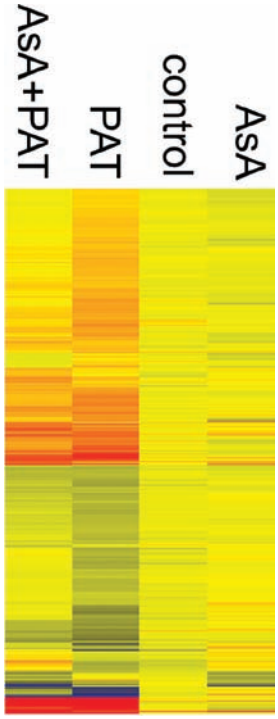

Figure 2. Hierarchical expression profiles after PAT and AsA treatment. Extracted genes were detected by ANOVA analysis. Each treatment condition is described in the text. Red lines indicate genes induced at least 1.5-fold greater than control. Conversely, blue lines indicate genes repressed at least 0.67-fold. Yellow lines indicate unchanged genes with expression changes between 0.67- and 1.5-fold.

following four conditions: control, AsA, PAT, and AsA + PAT. When changes were classified with respect to treatment condition, AsA treatment induced alteration in only 46 genes, whereas 568 genes were altered following PAT treatment. In the presence of both AsA and PAT, alterations in 290 genes were observed (Figure 2). This result suggests that AsA treatment substantially reduced the degree of influence of PAT in gene expression. These gene expression patterns were confirmed by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) (data not shown). Cellular iron transporter genes *FET3*, *FTR1*, *FET4*, *ARN1*, *2*, *3*, and *4*, which are described later, were selected, and *ACT1*, which codes actin, was used as an internal control. The results of RT-PCR corresponded to the DNA microarray.

***sod1* Mutant Is Suitable for the Analysis of PAT Toxicity.** When the PAT-responsive genes were classified according to their functional categories, the result indicated that PAT treatment causes gene expression changes in all categories and that AsA + PAT treatment produced a small number of changes in the same categories compared to PAT alone. This suggests that the influence of both PAT and AsA is not limited to a specific functional category (Table 1). The most highly induced and repressed genes are listed in Tables 2 and 3. The list of PAT-induced genes included the following: the aryl-alcohol dehydrogenase genes *AAD4* and *AAD6*, which belong to the family of oxidoreductase genes and are involved in the oxidative stress response; *FLR1*, which codes for a multidrug transporter; and YKL071W, which is a protein of unknown function. These significantly induced genes corroborate the result of a previous study[12] using yeast S288C strain. As a whole, oxidoreduction-reaction related genes were well represented (Table 4), although several genes of unknown functions are also listed in Table 2. Importantly, a number of genes that were highly repressed in response to PAT showed partial recovery by the addition of AsA (Table 3). Iwahashi[12] also reported that PAT-induced alterations in the expression of various stress responsive genes, such as *OYE2* and *OYE3*; NADPH oxidoreductase; *AAD4*, *AAD6*, and *AAD16*; and*SPS4* and *SPS100*, which code for DNA damage-response proteins; and in some of the unidentified genes, YKL070W and YKL071W. These genes were detected in the present study as well, with the exception of *SPS100*. Despite differences in yeast strain and growth conditions, the genes with significant expression changes were similar, so it is thought that *AAD4*, *AAD6*, *OYE3*, and *SPS4* are suitable as biomarkers for PAT toxicity.

PAT-repressed genes also included transcription factors, *SFG1*, *NRM1*, and *RPI1*; a cell cycle gene, *CLB1*; and cell wall-related proteins, *CWP1* and *GAS3*. It did not seem to be obvious that the expression changes in these genes have close correlation with oxidative stress. However, the expression of *FET4*, which is an iron−sulfur (Fe/S) cluster-related gene and plays a role in the low-affinity Fe(II) transporter,[26] was strongly repressed, and AsA could not restore its expression level. Fe/S cluster-related genes are sensitive to oxygen radicals, and repression of those genes is strongly indicative of oxidative stress.[27] Therefore, we next focused on and extracted Fe/S cluster and iron metabolism genes.

AsA Lessens the Repression of Fe/S Cluster Genes Except Transporters. Functional clustering highlighted a number of Fe/S cluster-related genes (Table 5). Reports already exist regarding the relationship between *sod1* mutant and *FET* genes. It was observed that the addition of iron improved growth in the *sod1* mutant.[28] This is due to the fact that *FET3* is induced in the *sod1* mutant. *FET3* codes for a multicopper ferroxidase, which is one of the components of the high-affinity Fe(II) transporter, and the cellular accumulation of iron enables resistance against oxidative stress.[28] Additionally, *FET4* is induced when other metal ions are added to the *fet3* mutant.[26,28] The double mutant, *fet3*/*sod1*, shows an increase in intracellular iron levels, suggesting that *FET4* is induced as an alternative iron uptake system, although the activity of the iron transporter Fet4p is lower than that of Fet3p.

In our present study, the expression of transporter component genes, *FET3* and *FTR1*, was repressed in all conditions compared with control. Even under AsA treatment alone, *FET3* and *FTR1* were significantly repressed, and a similar trend in gene expression was obtained from treatment with PAT alone. It appears that AsA treatment did not inhibit yeast cell growth, at least over the period of observation in our experiments (Figure 1b). Rather, under an AsA condition, it was thought that the gene repression of *FET3* and *FTR1* was due to a decreased need for activating the antioxidative system. However, PAT is known to cause oxidative stress; therefore, it was counterintuitive that both iron ion uptake genes, which assemble Fe/S cluster proteins and generate resistance against oxidative stress, were repressed. *FET3* is strongly regulated by transcription activators, Aft1 and Aft2.[29] In addition, mitochondrial inner membrane transporter Atm1 transmits a cellular iron uptake signal to Aft1/Aft2.[30] In our study, significant expression changes of *ATM1* or *AFT1* were not observed; it is thought that these genes did not have a crucial influence on cellular iron uptake and metabolism genes in our research condition. Chen et al.[31] indicated that there is an inverse correlation between Fet3−Ftr1 iron uptake and mitochondrial Fe/S cluster assembly. Several mitochondrial Fe/S genes, *MRS4*, *IBA57*, *ISU2*, and *NFU1*,[32−35] were induced by PAT, so it is

Table 1. Categories of ORFs Induced (>1.5-fold) or Repressed (<0.67-fold) by PAT and AsA Treatments

gene category	total number of genes detected by ANOVA	AsA treatment	PAT treatment	AsA + PAT treatment
metabolism				
amino acid metabolism (245)[a]	75	7	61	37
nitrogen and sulfur metabolism (97)	27	4	22	15
nucleotide metabolism (226)	34	0	23	13
phosphate metabolism (417)	47	0	30	12
C-compound and carbohydrate metabolism (505)	85	4	68	37
lipid, fatty acid, and isoprenoid metabolism (281)	41	0	27	13
metabolism of vitamins, cofactors, and prosthetic groups (164)	31	3	22	13
secondary metabolism (78)	18	3	14	8
energy (360)	60	3	45	28
cell cycle and DNA processing (998)	111	1	79	27
transcription (1022)	91	4	65	25
protein synthesis (477)	24	0	12	2
protein fate (1147)	124	0	83	27
protein with binding function (1039)	111	3	78	24
protein activity regulation (244)	31	0	20	7
cellular transport (1033)	137	16	93	47
cellular communication/signal transduction (234)	21	1	13	7
cell rescue, defense, and virulence (552)	124	16	91	54
interaction with the cellular environment (457)	61	11	45	26
development (70)	10	1	7	1
biogenesis of cellular components (859)	87	3	57	21
cell type differentiation (449)	46	0	32	11
unclassified proteins (1980)	235	10	168	94

[a] Numbers in parentheses indicate total ORFs in each category.

Table 2. Most Highly Induced ORFs by PAT Treatment[a]

systematic name	gene symbol	AsA treatment	PAT treatment	AsA + PAT treatment	description
YKL071W		0.95	79.78	64.76	protein of unknown function
YCL026C-A	*FRM2*	0.75	42.92	29.24	protein of unknown function
YFL056C	*AAD6*	0.62	30.29	22.08	putative aryl-alcohol dehydrogenase
YDL243C	*AAD4*	0.89	21.14	17.50	putative aryl-alcohol dehydrogenase
YBR008C	*FLR1*	1.04	17.86	15.55	plasma membrane multidrug transporter
YKL070W		0.90	15.09	10.76	protein of unknown function
YPL171C	*OYE3*	0.94	14.38	15.39	NADPH oxidoreductase containing flavin mononucleotide (FMN)
YDL218W		0.84	10.54	4.21	protein of unknown function; regulated by Azf1p
YLL056C		1.12	9.92	6.58	protein of unknown function
YCL026C-B	*HBN1*	1.06	9.52	9.60	protein of unknown function; similar to bacterial nitroreductases
YLR327C	*TMA10*	0.68	7.82	2.30	protein of unknown function that associates with ribosomes
YBR054W	*YRO2*	2.24	7.56	6.29	protein of unknown function; transcriptionally regulated by Haa1p
YFL057C	*AAD16*	0.76	7.26	6.34	putative aryl-alcohol dehydrogenase
YLL060C	*GTT2*	0.62	6.81	5.82	glutathione *S*-transferase
YKR076W	*ECM4*	0.78	6.37	5.35	omega class glutathione transferase
YCR021C	*HSP30*	0.55	5.70	2.76	hydrophobic plasma membrane localized stress-responsive protein
YLR303W	*MET17*	1.19	5.34	5.09	methionine and cysteine synthase
YKL086W	*SRX1*	0.76	5.25	3.18	sulfiredoxin, contributes to oxidative stress resistance
YLR346C		0.98	5.16	3.21	protein of unknown function
YNL277W	*MET2*	1.42	5.05	7.57	L-homoserine-*O*-acetyltransferase

[a] Values represent fold change relative to the control (= 1; DMSO treatment).

naturally thought that both *FET3* and *FTR1* were repressed. Because mitochondrial Fe/S genes also indicated a slight induction under an AsA−PAT condition, this might have disturbed the recovery of the iron transport system. Meanwhile,

Table 3. Most Highly Repressed ORFs by PAT Treatment[a]

systematic name	gene symbol	AsA treatment	PAT treatment	AsA + PAT treatment	description
YOR313C	*SPS4*	1.28	0.18	0.36	protein whose expression is induced during sporulation
YMR319C	*FET4*	0.96	0.18	0.23	low-affinity Fe(II) transporter of the plasma membrane
YLR413W		1.20	0.26	0.60	protein of unknown function
YKL096W	*CWP1*	1.37	0.27	0.79	cell wall mannoprotein
YMR006C	*PLB2*	1.12	0.29	0.62	phospholipase B (lysophospholipase)
YHL028W	*WSC4*	1.87	0.29	0.50	ER membrane protein; roles in the stress response
YOR315W	*SFG1*	1.15	0.29	0.52	nuclear protein, putative transcription factor
YNL112W	*DBP2*	0.94	0.29	0.36	essential ATP-dependent RNA helicase of the DEAD-box protein family
YJL200C	*ACO2*	1.33	0.30	0.60	putative mitochondrial aconitase isozyme
YNR009W	*NRM1*	1.02	0.32	0.66	transcriptional corepressor of MBF (MCB binding factor)-regulated gene expression
YGR108W	*CLB1*	1.28	0.33	0.52	B-type cyclin; accumulates during G2 and M
YPL061W	*ALD6*	1.04	0.34	0.58	cytosolic aldehyde dehydrogenase
YBR092C	*PHO3*	1.05	0.35	0.39	constitutively expressed acid phosphatase
YOR108W	*LEU9*	1.12	0.37	0.51	α-isopropyl malate synthase II (2-isopropyl malate synthase)
YJR016C	*ILV3*	1.41	0.39	0.80	dihydroxy acid dehydratase
YMR246W	*FAA4*	1.24	0.39	0.85	long-chain fatty acyl-CoA synthetase
YMR215W	*GAS3*	1.11	0.39	0.88	putative 1,3-*β*-glucanosyltransferase
YLR438W	*CAR2*	1.13	0.40	0.91	L-ornithine transaminase (OTAse)
YHR137W	*ARO9*	0.83	0.41	0.57	aromatic aminotransferase II
YIL119C	*RPI1*	1.06	0.41	0.38	putative transcriptional regulator

[a] Values represent fold change relative to the control (= 1; DMSO treatment).

Table 4. Oxidative Stress-Response and Resistance Genes Detected in This Study[a]

systematic name	gene symbol	AsA treatment	PAT treatment	AsA + PAT treatment	description
YCR107W	*AAD3*	0.96	1.56	1.63	putative aryl-alcohol dehydrogenase
YDL243C	*AAD4*	0.89	21.14	17.50	putative aryl-alcohol dehydrogenase
YFL056C	*AAD6*	0.62	30.29	22.08	putative aryl-alcohol dehydrogenase
YJR155W	*AAD10*	1.14	1.54	1.55	putative aryl-alcohol dehydrogenase
YNL331C	*AAD14*	0.94	1.62	1.16	putative aryl-alcohol dehydrogenase
YFL057C	*AAD16*	0.76	7.26	6.34	putative aryl-alcohol dehydrogenase
YMR038C	*CCS1*	0.89	1.46	1.41	copper chaperone for superoxide dismutase Sod1p
YGR088W	*CTT1*	1.54	1.25	1.94	cytosolic catalase T
YKL026C	*GPX1*	1.23	1.84	1.57	phospholipid hydroperoxide glutathione peroxidase
YBR244W	*GPX2*	0.78	1.59	1.22	phospholipid hydroperoxide glutathione peroxidase
YHR104W	*GRE3*	0.93	1.73	1.24	aldose reductase
YDR513W	*GRX2*	0.88	1.42	1.21	cytoplasmic glutaredoxin/thioltransferase/glutathione-dependent disulfide oxidoreductase
YJL101C	*GSH1*	0.91	2.16	2.36	*γ*-glutamylcysteine synthetase
YGR154C	*GTO1*	0.94	1.71	1.38	omega-class glutathione transferase
YLL060C	*GTT2*	0.62	6.81	5.82	glutathione *S*-transferase
YFL014W	*HSP12*	1.21	1.59	1.54	plasma membrane localized protein; regulated by the HOG and Ras-Pka pathways
YJL159W	*HSP150*	1.27	1.77	1.97	O-mannosylated heat shock protein
YHR179W	*OYE2*	0.89	2.53	2.74	widely conserved NADPH oxidoreductase
YPL171C	*OYE3*	0.94	14.38	15.39	conserved NADPH oxidoreductase
YER042W	*MXR1*	0.84	2.61	1.97	methionine-*S*-sulfoxide reductase
YCL033C	*MXR2*	1.08	1.73	1.33	methionine-*R*-sulfoxide reductase
YML007W	*YAP1*	0.80	1.54	1.16	basic leucine zipper (bZIP) transcription factor

[a] Values represent fold change relative to the control (= 1; DMSO treatment).

the AsA condition indicated a slight repression of mitochondrial Fe/S genes, albeit not a significant difference. However, under that condition, *FET3* was repressed as well as *AFT2*, and an inverse correlation was not observed. When taken together, each

Table 5. Iron Uptake System and Metabolism Genes Detected in This Study[a]

systematic name	gene symbol	AsA treatment	PAT treatment	AsA + PAT treatment	description
YPL202C	*AFT2*	0.77	1.23	0.85	iron-regulated transcriptional activator
YHL040C	*ARN1*	0.44	2.84	1.10	siderophore-iron transporter
YHL047C	*ARN2*	0.68	1.51	0.73	siderophore-iron transporter
YEL065W	*SIT1* (*ARN3*)	0.33	0.69	0.21	siderophore-iron transporter
YOL158C	*ENB1* (*ARN4*)	0.63	1.05	0.55	siderophore-iron transporter
YDR151C	*CTH1*	0.85	1.40	0.90	member of the CCCH zinc finger family
YLR136C	*CTH2* (*TIS11*)	0.38	1.36	0.42	mRNA-binding protein
YKR071C	*DRE2*	0.84	1.87	1.48	Fe/S cluster protein
YMR058W	*FET3*	0.63	0.71	0.36	ferro-O_2-oxidoreductase
YMR319C	*FET4*	0.96	0.18	0.23	low-affinity Fe(II) transporter of the plasma membrane
YOR382W	*FIT2*	0.35	1.27	0.34	mannoprotein involved in the retention of siderophore-iron in the cell wall
YLR214W	*FRE1*	0.28	0.86	0.33	ferric reductase and cupric reductase
YLL051C	*FRE6*	0.66	1.03	0.66	ferric reductase and cupric reductase
YER145C	*FTR1*	0.65	0.58	0.43	high-affinity iron permease
YJR122W	*IBA57*	0.80	1.48	1.16	mitochondrial iron−sulfur clusters assembly protein
YOR226C	*ISU2*	0.80	1.87	1.24	mitochondrial iron−sulfur clusters assembly protein
YKR052C	*MRS4*	0.70	1.68	1.02	iron transporter
YER042W	*MXR1*	0.84	2.61	1.97	methionine-*S*-sulfoxide reductase (MsrA)
YCL033C	*MXR2*	1.08	1.73	1.33	methionine-*S*-sulfoxide reductase (MsrB)
YGL091C	*NBP35*	0.94	1.70	1.41	iron−sulfur cluster binding protein
YKL040C	*NFU1*	0.96	1.52	1.32	protein involved in iron metabolism in mitochondria
YDR091C	*RLI1*	0.96	0.59	0.72	rron−sulfur containing RNase L inhibitor
YPL252C	*YAH1*	1.37	0.54	1.06	ferredoxin
YML007W	*YAP1*	0.80	1.54	1.16	leucine zipper transcription factor
YDR423C	*YAP2*	1.01	1.69	1.55	leucine zipper transcription factor

[a] Values represent fold change relative to the control (= 1; DMSO treatment).

response in gene expression involved in cellular iron transport is expected to follow one of three patterns as follows. (1) AsA condition: during the repression of both *AFT2* and mitochondrial Fe/S genes, *FET3* is influenced by *AFT2* rather than mitochondrial Fe/S genes; (2) PAT condition: during the induction of both *AFT2* and mitochondrial Fe/S genes, *FET3* is influenced by mitochondrial Fe/S genes rather than *AFT2*; (3) AsA + PAT condition: during the inverse trend of expression that consists of both *AFT2* repression and mitochondrial Fe/S gene induction, *FET3* is strongly repressed by both suppressive effects. It is thought that these expression changes strictly regulate intracellular iron concentration. Because the causal relationship between Aft and mitochondrial Fe/S to *FET3* regulation was still unclear, more research may be required.

On the other hand, although *FET4* was strongly repressed in the presence of PAT, it is not expected that AsA has an effect on *FET4* expression. Here, *ARNs*, which encode a siderophore-iron transporter,[36] showed unique expression changes. In particular, gene expression of both *ARN1* and *ARN2* was reduced by AsA, induced by PAT, and lessened in the AsA + PAT group. These two genes are highly induced by the overexpression of *AFT1*.[37] *AFT1* was not detected in this study, but *AFT2*, which is thought to have the same function, was detected. The expression pattern of *AFT2* was correlated with that of *ARN1* and *ARN2*. Therefore, these results suggest that the induction of *AFT2* also strongly affects the *ARN1* and *ARN2* genes, but not *ARN3* and *ARN4*. The expression of *ARN3* and *ARN4* indicated a trend similar to that of *FET3*−*FTR1*; additionally, they did not recover completely under the AsA−PAT condition. Collectively, these findings suggest that AsA treatment might not be able to recover the expression of many of the cellular iron transporter genes, although it is thought that the induction of *ARN1* and *ARN2* suppresses the arrest of the cellular iron transport system to some extent.

Iron ions introduced into cells accumulate in mitochondria for assembly of the Fe/S cluster.[38] In yeast cells, two SOD enzymes, Sod1 and Sod2, are very important for counteracting oxidative stress during the synthesis of normal Fe/S cluster proteins. Sod1 removes reactive oxygen species (ROS) and is localized to the intermembrane space (IMS) of mitochondria.[39] Because of this, it was expected that mitochondrial Fe/S cluster assembly genes would clearly reflect the influences of AsA or PAT by using the *sod1* mutant. In the present case, *MRS4*, which codes for a mitochondrial iron transporter, was induced by PAT treatment. The mitochondrial Fe/S cluster assembly genes *NFU1*, *IBA57*, and *ISU2* also showed the same trend. On the other hand, *YAH1*, which encodes a Fe/S protein ferredoxin,[40] was notably repressed by PAT treatment. Constructing a Fe/S cluster requires reduction by ferredoxin, so the repression of *YAH1* expression causes dysfunction in the composition of the Fe/S cluster and results in the accumulation of mitochondrial iron. The Fe/S cluster proteins are transferred from mitochondria to the cytosol and are processed by the cytosolic Fe/S protein assembly (CIA) machinery complex, which includes *NBP35*, for maturation.[38] Also, *MXR1* and *MXR2*, which encode methionine-*S*-sulfoxide reductases, protect the Fe/S protein assembly.[41,42] These

Table 6. DNA Damage-Response and Repair Genes Detected in This Study[a]

systematic name	gene symbol	AsA treatment	PAT treatment	AsA + PAT treatment	description
Induced by PAT Treatment					
YMR173W	*DDR48*	0.88	2.36	2.03	DNA damage-response protein
YER143W	*DDI1*	0.86	1.95	1.29	DNA damage-inducible v-SNARE binding protein
YDR078C	*SHU2*	1.05	1.60	1.16	DNA repair protein
YDR369C	*XRS2*	0.92	1.59	1.27	DNA repair protein
YDL059C	*RAD59*	0.90	1.58	1.37	DNA repair protein
YOR220W	*RCN2*	0.99	1.52	1.03	DNA damage-response protein
YGL163C	*RAD54*	1.04	1.52	1.54	DNA repair protein
YDR030C	*RAD28*	0.93	1.47	1.12	DNA repair protein
YNL250W	*RAD50*	0.75	1.47	1.10	subunit of MRX complex
YMR201C	*RAD14*	0.87	1.43	1.09	DNA repair protein
YBR136W	*MEC1*	0.91	1.39	1.09	genome integrity checkpoint protein
YDL200C	*MGT1*	0.91	1.38	1.01	DNA repair methyltransferase
YJR035W	*RAD26*	0.85	1.36	0.95	DNA repair protein
YER162C	*RAD4*	0.80	1.29	0.93	DNA repair protein
Repressed by PAT Treatment					
YOR313C	*SPS4*	1.28	0.18	0.36	DNA damage-response protein
YLR437C	*DIF1*	0.95	0.49	0.63	DNA damage-response protein
YPL127C	*HHO1*	1.33	0.50	1.33	histone H1; suppresses DNA repair
YBL009W	*ALK2*	1.00	0.51	0.95	protein kinase; DNA damage-response protein
YBL003C	*HTA2*	1.10	0.58	0.98	histone H2A, DNA repair
YGL021W	*ALK1*	1.03	0.59	0.71	protein kinase; DNA damage-response protein
YER095W	*RAD51*	1.00	0.60	0.90	strand exchange protein
YDR113C	*PDS1*	1.04	0.60	0.92	securin
YKL112W	*ABF1*	0.93	0.61	0.75	DNA binding protein; DNA repair
YJL092W	*SRS2*	1.05	0.62	0.92	DNA helicase and DNA-dependent ATPase involved in DNA repair
YMR078C	*CTF18*	0.91	0.63	0.77	subunit of a complex with Ctf8p; DNA repair
YHR154W	*RTT107*	0.93	0.66	0.94	DNA repair protein
YLL002W	*RTT109*	1.12	0.67	0.92	histone acetyltransferase critical for cell survival in the presence of DNA damage
YML058W-A	*HUG1*	0.93	0.71	0.62	DNA damage-response protein

[a] Values represent fold change relative to the control (= 1; DMSO treatment).

cytosolic protein-coding genes were induced under the PAT condition, and their induction was lessened under the AsA + PAT condition. Furthermore, *CTH1* and *CTH2*, which play a role in post-transcriptional iron regulation, were induced by PAT and repressed by AsA. *CTH2* is induced by *AFT2* deficiency and iron depletion;[43] however, PAT induced both *AFT2* and *CTH2* in our study. At the same time, the expression of *RLI1*, which codes for an RNase inhibitor and binds ribosomes,[44,45] was reduced by PAT treatment. The *RLI1*-deficient strain exhibits defects in stop codon recognition, resulting in abnormal translation of iron regulation proteins.[45] The transcription factors *YAP1*, which is directly involved in the oxidative stress response, and *YAP2*, which stabilizes proteins under oxidative stress conditions,[46,47] were also induced by PAT. Despite alterations of those genes, cell growth was suppressed under the PAT-exposed condition (Figure 1a), and these results suggest that abnormal alterations in iron regulation genes were caused by the normal antioxidative reaction not being effective against PAT toxicity.

What effect does ascorbic acid provide at this time? PAT repression of *FET4* and *FET3*−*FTR1* was not recovered, although a number of Fe/S cluster-related genes were recovered by AsA treatment. Furthermore, *ARN*s were repressed by AsA treatment regardless of PAT. Therefore, these alterations suggest that the influence of AsA is independent of the response against PAT toxicity with respect to iron transporters and that it does not contribute to the recovery from changes. Considering the recovery of Fe/S cluster assembly genes, AsA may affect those genes but not the iron transport genes. However, free iron-mediated oxidation is harmful,[48] and the overflow of iron can cause considerable stress to the cell if mitochondrial iron accumulation is not resolved. It is possible that AsA functions as a pro-oxidant under a free iron-rich condition, although it is better known as an antioxidant.[19−21] These characteristics work both ways against the oxidative stress response and are dependent on the intracellular iron ion status; therefore, this may be one of the reasons AsA cannot completely recover PAT toxicity.

Recovery of DNA Repair and Responsive Genes. PAT also causes DNA damage derived from oxygen radicals.[5,6] Consistent with this, a number of DNA repair and responsive genes were detected in this study (Table 6). With the detected *RAD*s, almost all of the genes were induced by PAT treatment, except *RAD51*, and reversed by cotreatment with AsA. A high expression level of *RAD51* is observed in the G1/S phase of the cell cycle,[49,50] so repression of this gene suggests G1/S phase dysfunction. *HUG1*,

Table 7. Cell Cycle Regulation Genes Detected in This Study[a]

systematic name	gene symbol	AsA treatment	PAT treatment	AsA + PAT treatment	description
YLR131C	*ACE2*	1.20	0.59	0.89	transcription factor expressed at early G1 phase
YJR092W	*BUD4*	1.08	0.61	0.91	protein involved in bud-site selection and required for axial budding pattern
YGL116W	*CDC20*	0.98	0.73	0.60	cell-cycle regulated activator of anaphase-promoting complex/cyclosome
YGR108W	*CLB1*	1.28	0.33	0.52	B-type cyclin
YPR119W	*CLB2*	1.04	0.46	0.60	B-type cyclin
YMR199W	*CLN1*	1.01	0.61	1.03	G1 cyclin
YPL256C	*CLN2*	1.34	0.66	1.86	G1 cyclin
YFR008W	*FAR7*	0.92	1.40	0.99	protein involved in G1 cell cycle arrest in response to pheromone
YIL131C	*FKH1*	0.96	0.56	0.78	forkhead family transcription factor
YOR372C	*NDD1*	1.15	0.63	1.35	transcriptional activator expressed at late S phase
YDR146C	*SWI5*	1.03	0.50	0.65	transcription factor expressed at the M/G1 phase
YDR451C	*YHP1*	1.05	0.59	1.00	homeodomain-containing transcriptional repressor
YML027W	*YOX1*	1.50	0.70	2.08	homeodomain-containing transcriptional repressor

[a] Values represent fold change relative to the control (= 1; DMSO treatment).

which functions to recover from transcriptional responses after DNA damage and replication arrest through the negative regulation of MEC1 effectors,[51] was repressed by PAT and remained repressed under the AsA + PAT condition. However, induction of *HUG1* requires an intact MEC1 pathway. The repression of *HUG1* implies that the MEC1 pathway was altered and remained damage response, so this suggests that the risk of DNA damage was not completely removed through the pathway, although *MEC1* recovered under the AsA + PAT condition. Meanwhile, many of the DNA repair genes, induced by PAT, were repressed by AsA + PAT treatment, and those alterations also indicate that AsA has considerable potential in lessening DNA damage.

AsA Affects the G1/S Phase Rather than the G2/M Phase. PAT causes G0/G1 and S phase arrest in murine skin cells and also causes G2/M arrest in Chinese hamster V79 cells.[4,5] In our study, both G1/S and G2/M arrest-related genes were detected (Table 7); they were repressed by PAT and recovered by the AsA + PAT condition with the exception of several genes. *FKH1*, which codes for a forkhead family transcription factor and negatively regulates the G2/M phase,[52] was repressed by PAT. In addition, *ACE2*, *CDC20*, *CLB1*, *CLB2*, and *SWI5*, which are regulated by *FKH1*,[53] showed the same trend following PAT treatment. However, compared with other cell cycle genes, these genes did not show robust recovery under the AsA + PAT condition. The *FKH1* deletion mutant induces *CLB2* and causes a slight increase in progression through the S and G2/M phases of the cell cycle.[54] However, in this study, the expression of *CLB2* corresponded to that of *FKH1*, suggesting that the repression of *CLB2* was caused by a factor other than *FKH1* regulation. The cooperative interaction of *FKH2* to the above-described genes is much stronger than that to *FKH1*,[52] so the alteration of *CLB2* might have been influenced by *FKH2* even though it was not detected. Of the genes in this category, only *FAR7* was induced by PAT. *FAR7* is a component of the complex that is induced by α-pheromone and causes G1 arrest.[55] Meanwhile, G1 cyclin *CLN1* and *CLN2*, which are expressed in late G1 phase and are involved in the G1/S phase transition, were repressed by PAT. The expression of *CLN*s is inhibited by various stressors, including oxidation or iron.[56] Therefore, the recovery of the expression patterns of *FAR7* and *CLN*s indicates the amelioration of G1/S phase arrest, which AsA treatment enabled. Taking alterations in gene expression as a whole, PAT treatment has an inhibitory influence on almost all of the cell cycle genes, suggesting that AsA treatment strongly affects G1/S phase genes rather than G2/M phase genes.

In conclusion, the *sod1* mutant was used to evaluate PAT toxicity, and our study highlighted expression changes in intracellular iron uptake and metabolism genes caused by oxidative stress. This is the first detailed comprehensive analysis of the relationship between AsA and PAT, and we notably revealed that the cellular iron transporter genes were not recovered by AsA treatment. These genes can be applied as biomarkers for future study. Because Fe/S cluster proteins play an important role in gene expression, it might be necessary to evaluate in detail the relationship between alterations in iron metabolism genes and cell cycle genes. Furthermore, the alterations in gene expression indicated that the risk of DNA damage and cell cycle arrest persists even with AsA treatment. To further examine the amelioration of PAT toxicity, it would be necessary to follow-up on the genes focused on in this study. In addition, evaluation of the relationship between AsA and PAT will be enabled by a more detailed verification of their effects on intracellular iron flux.

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