

The Viability Change of Yeast Cell Responding to Trehalose Accumulation and Maintaining Neutral Trehalase Activity under Extracellular pH Acidified by H₂SO₄

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Abstract *Saccharomyces cerevisiae* KNU5377 (KNU5377) and *S. cerevisiae* ATCC24858 (ATCC24858) were exposed to H₂SO₄ as a stress, which was added at various concentrations to a YPD media. The growth of KNU5377 was reduced to approximately 60% in the YPD media containing 4) mM sulfuric acid when compared to the non-stressed condition. When their growth was monitored during an overnight culture, two strains, KNU5377 and ATCC24858, could not grow when exposed to over 50 mM of sulfuric acid. After a short exposure to this acid for 1 h, KNU5377 exhibited stronger resistance against H₂SO₄ than ATCC24858. The neutral trehalase activity of KNU5377 unchanged despite under various concentrations of H₂SO₄. In contrast, that of ATCC24858 was much low at higher H₂SO₄ concentrations. Trehalose, a non-reducing disaccharide, was maximally accumulated after a short exposure to 60 mM H₂SO₄ for KNU5377, but it was reduced under more severe stressful conditions. These results suggest that KNU5377 should modulate the trehalose concentrations under the severe stress condition of high sulfuric acid concentrations. The most highly induced protein in the KNU5377 exposed to sulfuric acid was found to be an approximately 23 kDa protein, which was revealed to be the 60S large subunit ribosomal protein, L13, by FASTA search results.

Key words: *Saccharomyces cerevisiae*, stress response, trehalose, ribosomal protein L13

Introduction

Under both environmental stressful and normal growth conditions, cells synthesize a set of proteins referred to as heat shock proteins (Hsps), which play a crucial role in facilitating the stabilization of unfolded proteins, maintaining the trans-

formed refolded proteins and rearrangements [4]. Consequently, triggered Hsps protect cells against elevated temperatures and other environmental stresses [18]. As another stress protecting agent, trehalose (α -D-glucopyranosyl-(1-1)- α -D-glucopyranoside) is a non-reducing disaccharide composed of two glucose rings found in bacteria and insects [5]. In *Saccharomyces cerevisiae*, this molecule is a reserve carbohydrate and plays a role as a chemical chaperone binding to proteins or other critical cellular targets such as the biological membranes in order to prevent their denaturation [2,9,24,25]. Increased trehalose concentrations are a response to elevated temperatures, ethanol and hydrogen peroxide [1]. There is still some controversy in that the correlation between cell tolerance and trehalose accumulation is not always paralleled under some stress conditions [17]. Nwaka et al. reported that the trehalose-degrading enzyme, NTH1, lacking a *nth1* mutant retained a high level of trehalose but actually had a lower thermotolerance [15,16]. Although this disaccharide has a protective role binding to the native protein, the stabilization of denatured proteins by this molecule may subsequently impede the ability of the Hsps as molecular chaperones [22]. Therefore, during the recovery from heat shock, trehalose must be hydrolyzed by trehalase in order, to liberate the cellular structures [23]. Trehalose degradation is mediated by two types of trehalase, which are composed by a cytosolic neutral enzyme encoded by *NTH1* and a vacuolar acid enzyme encoded by *ATH1*. With a high homology to *NTH1*, it is unclear whether or not the product of *NTH2* has trehalase activity [17]. *NTH1*, a neutral trehalase, controls trehalose hydrolysis in response to various stress conditions in *Saccharomyces cerevisiae* [26]. This phenomenon, controlling trehalose accumulation and hydrolysis of the molecule, should also be important in other stressful environmental conditions.

In contrast, the exposure of *Saccharomyces cerevisiae* cells to weak organic acids such as propionic, sorbic and benzoic acid induces an ATP-binding cassette (ABC) trans-

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porter *Pdr12* in the plasma membrane to confer weak acid resistance on the cells by the efflux of water-soluble carboxylate anions [19]. In the case of inorganic acids, activation of the plasma membrane H^+ -ATPase was attributed to a lowering of the extracellular pH, a pH 3.5 acidified by 50 mM acetic acid or HCl, and a resulting decrease in the cytosolic pH. In addition, the vacuolar pH was accompanied with that decrease. These results suggest that a low extracellular pH can affect the internal pH, both the cytosolic and vacuolar pH, to make the cells control the pH homeostasis [3]. Despite these studies, the cellular response to low extracellular pH caused by various organic and inorganic acids in the stress response of *Saccharomyces cerevisiae* is poorly understood.

In this work, the change in trehalose accumulation and the activity of the trehalose hydrolyzing enzyme, neutral trehalase, and the comparison of the resistance under various H_2SO_4 concentrations was investigated both in this strain *Saccharomyces cerevisiae* KNU5377 and a reference strain *S. cerevisiae* ATCC24858. In addition, the amino acid sequencing data of a 23 kDa protein, which was highly expressed after exposure to sulfate, was also reported.

Materials and Methods

Strains and Growth condition

A yeast isolate from Korean soil, named as *Saccharomyces cerevisiae* KNU5377 [11,12], was grown on a YPD (Yeast extract 1%, Peptone 1% and Dextrose 1%) agar plate at 30°C and stored into refrigerator until needed. 1% of exponentially growing cells inoculated on the fresh YPD media with or without H_2SO_4 were cultivated until the mid-log phase. Under the same stress condition, a reference strain, *S. cerevisiae* ATCC24858, was used as a comparison.

Measurement of cell viability

Two strains grown to $OD_{600}=1$ on the YPD media were treated with 0, 20, 40, 60, 80, 100 and 120 mM H_2SO_4 for 1 h, and subsequently the serial 10-fold diluted cells were plated in triplicate onto the YPD media in order to monitor cell viability. After cultivation at 30°C for 2 days, a number of colonies formed under various concentration of the chemical were calculated as a percentage by comparing them with the colonies formed in the 0 mM H_2SO_4 condition, which is defined as 100%. Moreover, the dose-dependent death kinetics of the two strains were measured after treatment with 30 and 50 mM H_2SO_4 .

Extraction and assay of trehalose

The cellular trehalose was extracted from the cells and concentrated as described [10]. The exponentially growing cells were treated with an appropriate H_2SO_4 concentration and washed with pre-cooled sterilized distilled water three times to remove the residual glucose. After adding 1 ml of sterilized distilled water to the washed cells, the cells were

incubated at 95°C for 20 min, and the supernatant was obtained by centrifugation. The trehalose concentration was determined by its enzymatic hydrolysis to glucose by trehalase (Sigma) after being incubated at 37°C for 12 h, and a glucose assay kit was used to measure the released glucose (Boehringer Mannheim). To determine how much H_2SO_4 stimulated the intracellular accumulation of trehalose, the preexisting glucose was subtracted from the total glucose concentration. The trehalose concentration was defined as the number of μg of trehalose per dried cell weight.

Neutral trehalase activity assay

The cells collected after the H_2SO_4 stress treatment were washed with pre-cooled sterilized and distilled water three times, and 500 μl of a lysis buffer (50 mM imidazole-HCl, pH 7.0 and 1 mM phenylmethyl-sulphonyl fluoride) and 1 volume of glass beads (425~600 microns, Sigma) were added sequentially. After cell disruption by micromixing at 4°C, the crude trehalase fraction of the supernatant was obtained by centrifugation. The reaction was initiated by adding the crude enzyme solution to a reaction mixture consisting of 50 mM phosphate buffer pH 7.2 and 500 mM of trehalose. After incubation for 20 min at 37°C, the glucose released from the trehalose was determined by a D-glucose assay kit (Boehringer Mannheim). The total protein concentration was assayed by a protein assay kit based on the Bradford method (Bio-Rad). One unit of neutral trehalase was defined as the amount of enzyme that, produces 1 μmol of glucose per min at 37°C at pH 7.2.

SDS polyacrylamide gel electrophoresis

The protein extract was prepared from 20 ml of the mid-log cultured cells after the H_2SO_4 treatment. The cells harvested by centrifugation were washed three times with sterile distilled water and suspended in an equal volume of the lysis buffer (50 mM imidazole-HCl, pH 7.0 and 1 mM phenylmethyl-sulphonyl fluoride). An equal volume of glass beads was added and the cells were disrupted by vortex mixing at 4°C. After centrifugation at 8000 rpm for 15 min, the supernatant was analyzed on 12% polyacrylamide gels according to the method reported by Lammler [13] to monitor the protein profiles under the stress condition.

Pulse-chase labeling of stress-induced proteins

Pulse-labeling of the yeast cellular proteins was achieved by adding a radiolabeled amino acid to the exponentially growing cells using 1Ci L-[^{32}S]-methionine per 10^6 cells in the 10-min pulse-labeling experiments under 50 mM H_2SO_4 . Radioactive incorporation was quenched by adding a protein synthesis inhibitor, cycloheximide (50 $\mu g/ml$), and the cells were then washed three times with 20 mM phosphate buffer, pH 7.0. One volume of 20 mM phosphate buffer, pH 7.0 was added to cell pellets and the glass beads (425~600 microns, Sigma). The cells were disrupted by vigorous vortex mixing for 1~2 min and subsequently placed on ice

water for 10 min, three times. The glass beads were removed by centrifugation, and the protein profiles were then examined by a routine SDS-PAGE method. The radioactive gels were dried on 3MM filter paper (Whatman) under vacuum at 80°C for 60~90 min. The labeled gels were exposed to X-ray film (Fuji).

Purification of target proteins

The proteins were fractionated on 10% SDS-PAGE gels to purify the H₂SO₄ stress-induced proteins from the SDS-PAGE gel. The 23 kDa protein band was cut with razor blades, pooled and chopped into smaller pieces (~4 mm). The proteins (about 500 μg per sample) were electroeluted from the pieces in an Elutrap device (Schleicher & Schuell) for 5~6 h at 250 volts in 25 mM N-ethylmorpholine (pH 8.5) and the quantity was estimated after SDS-PAGE after comparing them with a set of Coomassie-stained bovine serum albumin standards.

Sequencing of induced proteins

The elutant containing the 23 kDa protein was concentrated by lyophilization and electrophoresed in a 12% and 10% of SDS-PAGE gel, which was then transferred to a Sequi-blot PVDF (polyvinylidene difluoride) membrane (Bio-Rad). The protein bands were visualized with Coomassie R-250 staining and sequenced by automated Edman degradation (Applied Biosystems Procise) at this university.

Results and Discussion

Growth inhibition of sulfuric acid

Sulfuric acid, a strong inorganic acid, is extremely corrosive, and easily dissociates H⁺ and SO₄²⁻ in water resulting in a lower environmental pH. These characteristics were applied to acid hydrolysis of the cellulosic biomass whose hydrolysates are used as a substrate by the fuel alcohol fermenting yeast, *Saccharomyces cerevisiae*, to produce an alternative energy resource, fuel alcohol [21]. However, in fermenting ethanol, the mechanism of the stress response in yeast cells is not well understood.

The precultured cells were inoculated on a glucose rich media, YPD, containing 0, 10, 20, 30, 40, 50 and 60 mM H₂SO₄. The growth of the two strains was dose-dependently reduced, but this response did not increase at H₂SO₄ concentrations over 50 mM (Fig. 1). Under 40 mM H₂SO₄, cell growth was reduced to 60% of the growth observed in the unstressed condition.

Sensitivity test against sulfuric acid

To examine the effect of H₂SO₄ toxicity, the two strains were exposed to 0, 20, 40, 60, 80, 100, 120 and 140 mM of H₂SO₄ for 1 h. A reference strain of *S. cerevisiae* ATCC24858 was able to survive at 80 mM H₂SO₄ only for 1 h. However, this *S. cerevisiae* KNU5377 could survive at 140 mM

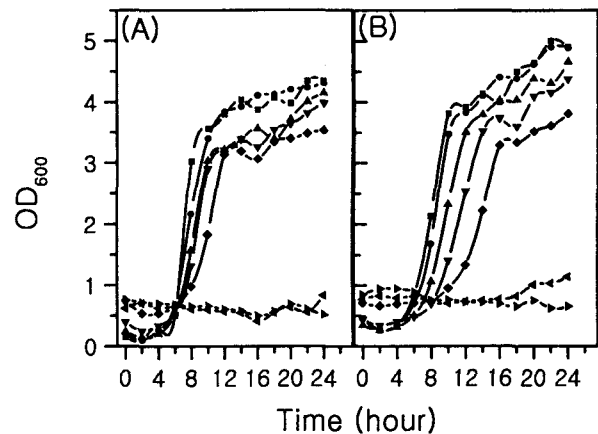


Fig. 1. Growth curve under various H₂SO₄ concentrations. Pre-cultured cells of *Saccharomyces cerevisiae* ATCC24858 (A) and *S. cerevisiae* KNU5377 (B) were inoculated in YPD containing 0 (■), 10 (●), 20 (▲), 30 (▼), 40 (◆), 50 (◄) and 60 mM (◄) of sulfuric acid.

H₂SO₄, indicating that the resistance of a reference *S. cerevisiae* strain, ATCC24858, to H₂SO₄ against increased concentration H₂SO₄ were significantly lower than KNU5377 (Fig. 2). Moreover, the results of the death kinetics of both strains determined under a sub-lethal dose of H₂SO₄, 30 mM and 50 mM, suggest that KNU5377 is acid tolerant by showing 50% survival after being exposed to H₂SO₄ for 1 h (Fig. 3).

Accumulation of trehalose

Treatment with elevated H₂SO₄ concentrations caused trehalose accumulation in the ATCC24858 cells (Fig. 4). In contrast, KNU5377 showed the maximum accumulation of this disaccharide at 60 mM H₂SO₄, but the level of accumulation reduced at higher H₂SO₄ concentrations. These results suggest that a considerable trehalose accumulation in the cells should not be always parallel to the stress-

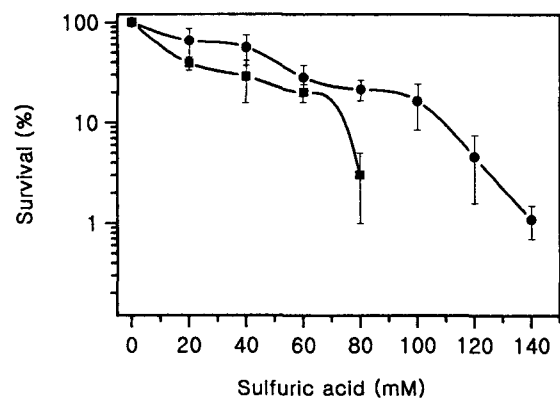


Fig. 2. Sensitivity test against H₂SO₄. Exponentially growing cells were exposed to the indicated H₂SO₄ concentration for 1 h. (■), *S. cerevisiae* ATCC24858; (●), *S. cerevisiae* KNU5377.

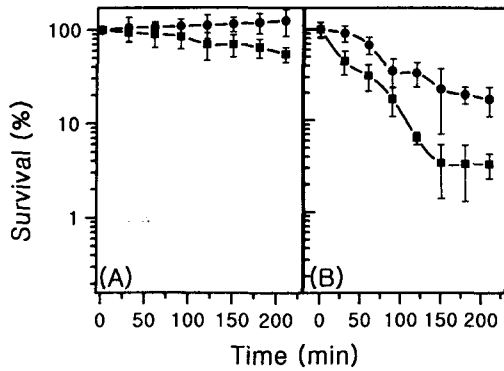


Fig. 3. Death kinetics after exposure to H₂SO₄. Mid-log cultured *S. cerevisiae* ATCC24858 (-■-) and *S. cerevisiae* KNU5377 (-●-) were aliquotted and treated for the indicated time with 30 (A), 50 mM (B) of H₂SO₄. The cells were picked up every 30 min and diluted and plated onto the YPD to obtain the percentage viability compared to the untreated control.

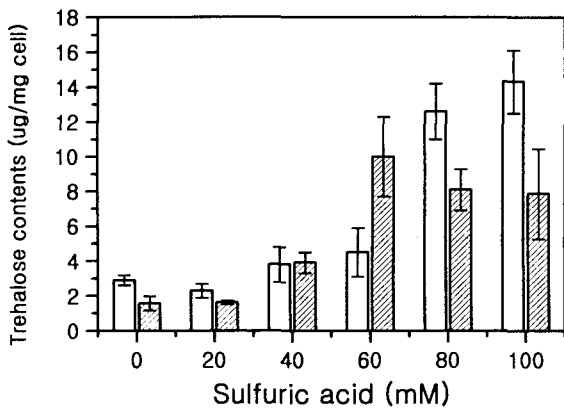


Fig. 4. Changes of trehalose accumulation at various H₂SO₄ concentrations.

After being exposed to the indicated H₂SO₄ concentrations for 1 h, the trehalose contents were extracted and determined by an enzymatic glucose assay. Open bar, *S. cerevisiae* ATCC24858; closed bar, *S. cerevisiae* KNU5377.

tolerance, and even though the level of trehalose was low in KNU5377, its roles were maintained at severe stress conditions.

Trehalose is known to protect native proteins and to suppress the aggregation of denatured proteins caused by stress conditions. After heat shock, trehalose is normally degraded to fulfill the recovery system of molecular chaperones, which promote the rearrangement of denatured proteins. However, maintaining a high level of trehalose interferes with the refolding of aggregated proteins [23]. Indeed, in KNU5377, trehalose was degraded by neutral trehalase in high H₂SO₄ concentrations. These results concur with an earlier report showing that *nth1* mutants, lacking the trehalose-degrading enzyme, Nth1, retained high levels of trehalose, but a lower thermotolerance [15,16]. With this in mind, it appears that the correlation between the cell tolerance and trehalose

accumulation is not always parallel [17].

The activity of neutral trehalase

The trehalose concentration in ATCC24858 increased during the more severe stress conditions. However, its neutral trehalase activity increased less at higher H₂SO₄ concentrations. In contrast, the neutral trehalase activity was higher than that of the normal condition in KNU5377 (Fig. 5). The difference in the neutral trehalase activity between ATCC24858 and KNU5377 at elevated H₂SO₄ concentrations means that the modulation of trehalose concentration in response to inorganic H₂SO₄ is strain-dependent. To liberate the trehalose, the bound proteins or cellular compartments required the sulfuric acid-induced expression of the Hsps as molecular chaperones for the target proteins. This results in KNU5377 acquiring acid tolerance and the trehalose rearrangement, as shown in Fig. 3.

Identification of the proteins induced by exposing to sulfuric acid

Exposure to sub-lethal and severe H₂SO₄ concentrations resulted in the induction of proteins, approximately 160, 90, 70, 60, 40 and 23 kDa. The 160 and 90 kDa proteins were over-expressed only in the KNU5377 cells by over 90 mM H₂SO₄ for 1 h and the others were observed in both strains (Fig. 6). The 160 kDa protein was stress-induced only in the KNU5377 cells and the 23 kDa protein was induced in both strains through the 10 min pulse-labeling experiment (Fig. 7). The electroeluted 23 kDa protein was subsequently determined for N-terminal amino acid sequencing. A database search with the FASTA network service indicated that the 23 kDa protein was a 60S large subunit ribosomal protein, L13A (RPL13A), or L13B (RPL13B), which was encoded by YDL082w and YMR142c, respectively (Table. 1).

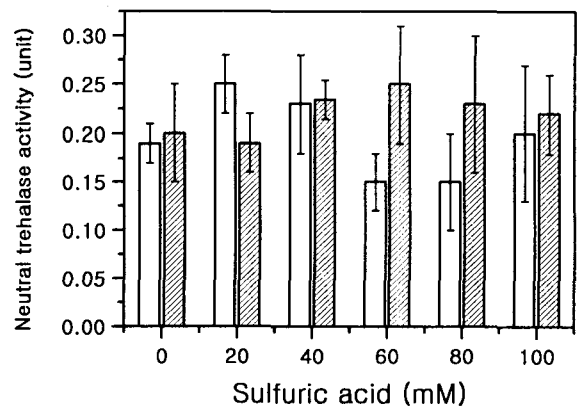


Fig. 5. Neutral trehalase activity assay.

After being exposed to the indicated concentration of sulfuric acid for 1 h, the neutral trehalase activity was calculated from the crude enzyme fraction of cell extract. Definition of the units : one unit of neutral trehalase is defined as the amount of enzyme which produces 1 μmol glucose/min at 37°C at pH 7. Open bar, *S. cerevisiae* ATCC24858; closed bar, *S. cerevisiae* KNU5377.

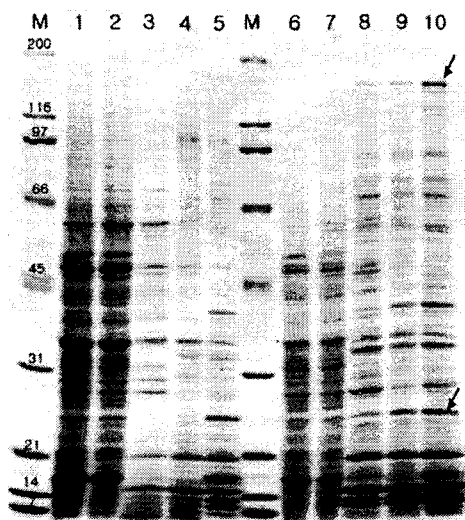


Fig. 6. Induced protein profiles after exposure to H₂SO₄. Exponentially growing *S. cerevisiae* ATCC24858 (lane 1 to 5) and *S. cerevisiae* KNU5377 (lane 6 to 10) were exposed to 0 mM (lane 1, 6), 30 mM (lane 2, 7), 90 mM (lane 3, 8), 150 mM (lane 4, 9) and 300 mM (lane 5, 10) H₂SO₄. It showed that the approximately 90, 70, 60, 40 and 23 kDa proteins were induced in both yeast cells, but the 160 kDa protein (upper arrow) was only expressed in *S. cerevisiae* KNU5377.

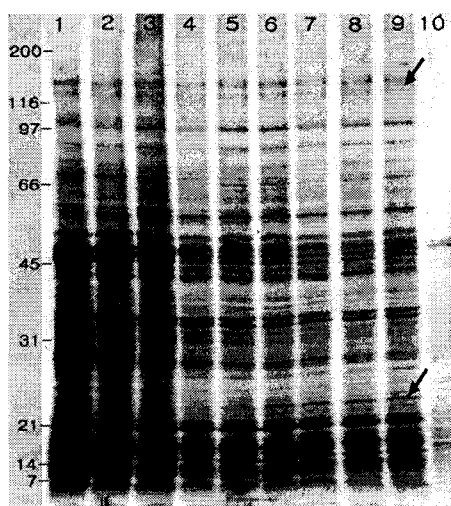


Fig. 7. Autoradiography. After 10 (1), 20 (2), 30 (3), 40 (4), 50 (5), 60 (6), 70 (7), 80 (8) and 90 (9) min with ³⁵S-Methionine under 50 mM H₂SO₄, about 160 and 23 kDa proteins were highly expressed in *S. cerevisiae* KNU5377. Lane 10 shows the effect of 200 μg/ml of cycloheximide added to the cells to inhibit protein synthesis for 90 min under the stress conditions.

As is well known, a yeast ribosome has 78 ribosomal proteins (RPs), including 32 different small ribosomal subunit proteins and 46 large ribosomal subunit proteins, which are assembled into ribosomes in the nucleolus [21]. The transcriptional regulation of a number of RP-genes has been modulated by the upstream activator sequence (UAS), which are promoter regions of most RP-genes with a similar architecture [20]. Two Rap1 binding sites, followed by one T-rich stretch and a TATA box, are required for the efficient transcriptional regulation of the RP-genes [6]. The RP-genes display variations in their transcription levels as a response to changes in the growth conditions and/or heat shock shifting to 36°C [7,8]. In addition, sporulation and tetrad analysis of the heterozygous deletion mutant revealed that YDL082w was a non-essential gene together with YDL086w and YDL085w [14]. The role of these proteins in terms of the stress response is unclear. However, their function as a structural constituent of a ribosome may be to endow the cells with more effective protein synthesis under stressful conditions. In conclusion, the 160 and 90 kDa proteins as well as this amino acid sequenced 23 kDa protein should be further investigated in order to determine their exact role in the yeast cell, *Saccharomyces cerevisiae* KNU5377.

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Table 1. Sequence comparison of approximately 23 kDa protein with RPL13A and RPL13B.

Sequence of 23 kDa protein	1 MAEPVNIPKLA 10
Amino acid position in RPL13A	1 M*A*ISKN*L*P*IL*KNHFRKHWQE 20
Amino acid position in RPL13B	1 M*A*ISKN*L*P*IL*KNHFRKHWQE 20

* Matching amino acids were marked as asterisks.

- additional elements apart from binding sites for Abf1p or Rap1p. *Nucl. Acids Res.* **23**: 1475-1480.
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