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Vacuolar H^+-ATPase, but not mitochondrial F_1F_0-ATPase, is required for aluminum resistance in *Saccharomyces cerevisiae*

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Abstract

It was recently shown that vacuolar ATPase and mitochondrial F_1F_0-ATPase activities are induced by aluminum (Al) in an Al-resistant cultivar of wheat, suggesting that induction of these enzymes could be an adaptive trait involved in Al resistance. To test this hypothesis, we used the *Saccharomyces cerevisiae* model system. In yeast, unlike wheat, the activity, transcript and protein levels of mitochondrial F_1F_0-ATPase, but not vacuolar ATPase, are induced by Al, while plasma membrane P-ATPase activity is inhibited. However, yeast vacuolar ATPase mutant strains are hypersensitive to Al, while F_1F_0-ATPase mutant strains exhibit wild-type growth. These data suggest that vacuolar ATPase activity is involved in Al resistance, with ATP required for this activity supplied by mitochondrial F_1F_0-ATPase or fermentation. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Aluminum stress; Vacuolar H^+-ATPase; Mitochondrial F_1F_0-ATPase; Al resistance; *vma1,2* mutation; *atp1,2* mutation

1. Introduction

Aluminum (Al) is the most abundant metal in the Earth's crust, and under acidic soil conditions speciation of Al compounds naturally present in soil changes in such a way that Al becomes soluble and toxic. Although many mechanisms have been proposed to account for Al resistance in crop species (see for example [1]), few have been tested directly. We recently showed that vacuolar ATPase and mitochondrial F_1F_0-ATPase activities are induced by Al stress in an Al-resistant cultivar of wheat [2]. The synthesis of peripheral ATPase subunits (RMP51) is induced by Al in a dose- and time-dependent manner, and protein levels decline after removal of Al [3]. Al-induced synthesis of these subunits segregates with Al resistance in progeny derived from a cross between sensitive and resistant cultivars [4]. This suggests that induction of V-ATPase and F_1F_0-ATPase may be an adaptive trait involved in Al resistance.

To further our understanding of Al resistance, we have developed a testable hypothesis describing the role of V-ATPase and F_1F_0-ATPase in Al resistance (Fig. 1). Based on our model, inhibition of the plasma membrane ATPase (P-ATPase) by Al would cause inhibition of secondary active transport processes, proton accumulation in the cytoplasm and decreased cytoplasmic pH. To counteract this effect, we predict an increase in activity of the vacuolar ATPase (V-ATPase), which would transport the excess protons into the lumen of the vacuole, thus alleviating the stress caused by cytoplasmic acidosis. Increased V-ATPase activity could also contribute to energizing the tonoplast membrane. It is possible that maintaining the tonoplast membrane electrochemical potential could provide energy required for a secondary active transport process involved in Al resistance. The ATP required for these processes could be provided by increased activity of ATP synthase (F_1F_0-ATPase).

To test this model directly, we used yeast as a model system. Although yeast and plants do not necessarily respond to stress in a similar fashion, yeast has the advantages of rapid, simple transformation, short life cycle and amenability to manipulation. This allows rapid hypothesis testing before, or while, more arduous experiments in plants are undertaken.

Here we examined the response of wild-type yeast to Al and tested the effect of mutations in V-ATPase and F_1F_0-ATPase subunits on Al resistance. If our model is correct,

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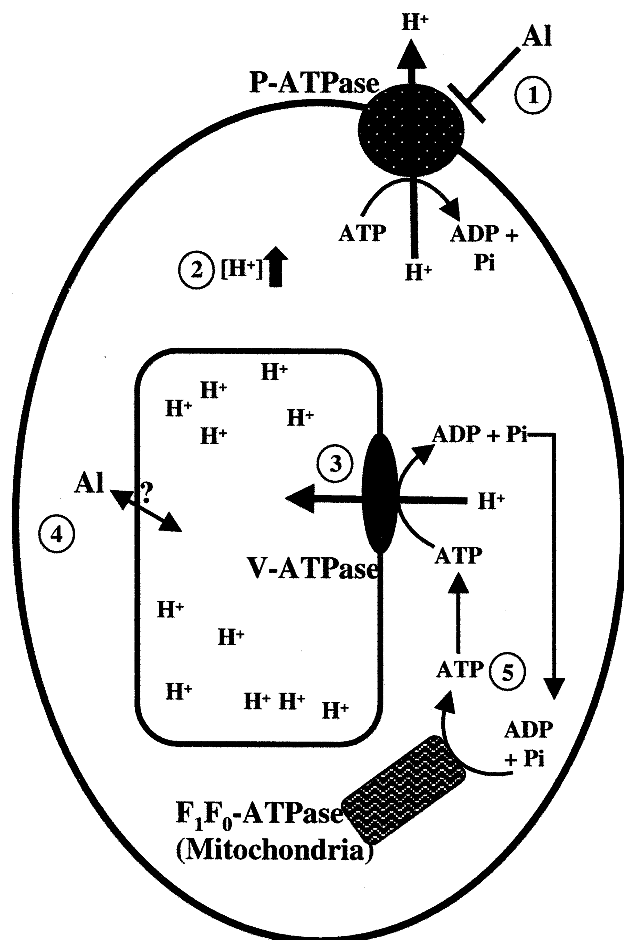

Fig. 1. Model representing the energy balance hypothesis for V-ATPase and F_1F_0-ATPase-mediated Al resistance in wheat. (1) Inhibition of the plasma membrane ATPase (P-ATPase) by Al. (2) Proton accumulation in the cytoplasm causes cytoplasmic acidosis. (3) Increased activity of the vacuolar ATPase (V-ATPase) could transport the excess protons into the lumen of the vacuole, alleviating the stress caused by decreased cytoplasmic pH and energizing the tonoplast membrane. (4) It is possible that maintaining the tonoplast membrane potential could provide energy required for a secondary active transport process involved in a hypothetical Al resistance mechanism. (5) The energy required for these processes could be provided by increased activity of ATP synthase (F_1F_0-ATPase).

then inhibiting the activity of the V-ATPase should cause increased sensitivity to Al. Inhibiting the activity of the F_1F_0-ATPase may have a lesser effect on Al resistance, since yeast, unlike plants, can produce ATP to support V-ATPase activity via fermentation.

2. Materials and methods

2.1. Media and culture conditions

Yeast strains (Table 1) were maintained on standard media as described by Sherman [5]. For experiments using Al, low pH, low phosphate (LPP) medium [6] was used. This medium is synthetic complete (SC) minimal medium (0.67% yeast nitrogen base, 2% glucose) with K_2HPO_4 reduced to 100 μM, KCl added to 4.9 mM and the pH reduced to 3.5 to maximize solubility, and therefore toxicity, of Al.

2.2. ATPase activity measurements

Seed cultures (3 ml in SC) of wild-type yeast were inoculated from YPD plate stocks and incubated for 16–24 h at 30°C. Cells were harvested by centrifugation, washed three times and resuspended in 1 ml of sterile water. This cell suspension was used to inoculate triplicate 3-ml cultures of LPP medium (pH 3.5) containing 0–15 μM $AlCl_3$ to a starting OD_{600} of approximately 0.01 and cultures were incubated for 16–24 h at 30°C (final OD_{600} approximately 0.9). Each 3-ml culture was harvested by centrifugation and spheroplasts prepared as described by Uchida et al. [7]. Cell suspensions were then sonicated at low intensity (Branson Sonifier, 10% output) for 30 s to gently lyse cells. Cell debris was removed by centrifugation and the resulting supernatant used for enzyme assays. Quantitation of protein in cell extracts was performed using the Bradford assay [8]. ATPase activity was then assayed as described by Briskin et al. [9].

2.3. Complementation of ATPase mutations

Yeast strains carrying *LEU2* insertion mutations in *VMA1* and *VMA2*, as well as plasmids carrying the complementing genes, were provided by T. Stevens (University of Oregon, OR, USA). Strains carrying null deletion mutations of *ATP1* and *ATP2* and plasmids carrying the complementing genes were provided by D. Mueller (Chicago Medical School). In order to select for transformation of the plasmids in the appropriate mutant strains, *ATP1* and *VMA2* were recloned into pRS314 (*TRP1*) and pRS316 (*URA3*), respectively, using standard molecular biology techniques [10]. The complementing constructs were then transformed into appropriate yeast strains using the lithium acetate method [11].

2.4. Al dose response experiments

Inoculum for each culture was prepared as described above and used to inoculate triplicate 3-ml cultures of SC medium containing 0–150 μM $AlCl_3$ to a starting OD_{600} of approximately 0.01. Cultures were incubated for 16–24 h at 30°C and the final OD_{600} of each culture was measured in a 96-well plate (Costar) using a Spectromax Plus plate reader and Softmax Pro v 1.1 software (Molecular Devices). For comparison, the OD_{600} values for each strain were expressed as a percentage of the control value for that strain.

Table 1
Yeast strains used in this study

Strain	Genotype	Source
SF838-1D	*MATα, ade6, his4-519, leu2-3, 112, ura3-52, pep4-3*	[30]
vma1	Isogenic to SF838-1D except *vma1Δ::LEU2*	[31]
vma2	Isogenic to SF838-1D except *vma2D:LEU2*	[32]
vma1/VMA1	Isogenic to *vma1* except carrying wild-type *VMA1* on plasmid pRS316 (*URA3*)	This study
vma2/VMA2	Isogenic to *vma2* except carrying wild-type *VMA2* on plasmid pRS316 (*URA3*)	This study
W303-1A	*MATα, ade2-1, his3-1, 15, leu2-3, 112, trp1-1, ura3-1*	[33]
W303-Δα	Isogenic to W303-1A except null deletion mutation in *ATP1*	[33]
W303-Δβ	Isogenic to W303-1A except null deletion mutation in *ATP2*	[33]
W303-Δα/*ATP1*	Isogenic to W303-Δα except carrying wild-type *ATP1* on plasmid pRS314 (*TRP1*)	This study
W303-Δβ/*ATP2*	Isogenic to W303-Δβ except carrying wild-type *ATP2* on plasmid pRS316 (*URA3*)	This study

2.5. Immunoblotting

Total yeast proteins were separated by SDS–PAGE [12] and electroblotted onto nitrocellulose (0.45 μm, Bio-Rad Laboratories) membranes using the Mini Trans-Blot Cell (Bio-Rad Laboratories) according to the manufacturer's instructions. Membranes were blocked and probed as directed in the Lumi-glo Chemiluminescent Detection System manual (Kirkegaard and Perry Laboratories). Polyclonal antibodies raised against the α and β subunits of the yeast F_1-ATPase were provided by A. Lewin, University of Florida, FL, USA. Antibodies raised against the B subunit of the *Vigna radiata* V-ATPase were provided by M. Maeshima, Nagoya University, Japan.

2.6. RNA isolation, Northern hybridization and analysis

RNA was isolated from yeast cultures (approximately 5×10^7 cells) using the RNeasy kit (Qiagen) according to the manufacturer's directions. Northern transfers were carried out using GeneScreen Plus (DuPont) membranes according to the manufacturer's directions. Probes for Northern blots were isolated from pRS316VMA2 and pRS316ATP1 by digestion with appropriate restriction enzymes (Amersham Pharmacia Biotech) and inserts were isolated from agarose gels using the QiaQuick gel extraction kit (Qiagen). Probes were then prepared by random priming [10]. Membranes were prehybridized, hybridized and washed according to the manufacturer's directions, followed by exposure to storage phosphor screens (Molecular Dynamics). Phosphor screens were developed using a Phosphorimager 445SI and the resulting images analyzed using ImagequaNT v 4.2 software. For figure preparation, representative lanes of triplicate samples were selected from the same blot and compiled using Adobe Photoshop® v 5.5.

3. Results and discussion

3.1. Response of yeast ATPases to Al

To confirm that yeast is a suitable model for investigating the effect of Al on ATPases in plants, the responses of ATPases to Al in wild-type yeast Al were examined (Fig. 2). F_1F_0-ATPase activity increased to 0.225 nmol Pi mg^{-1} min^{-1} (580% of control) at 2.5 μM $AlCl_3$. Although the Al-induced stimulation declined to 0.137 nmol Pi mg^{-1} min^{-1} (340% of control) from 5 to 10 μM $AlCl_3$, F_1F_0-ATPase activity was still 0.151 nmol Pi mg^{-1} min^{-1} (380% greater than control) at 15 μM, similar to what has been observed in wheat [2]. P-ATPase activity also showed an effect similar to what has been observed in plants [2,13–16], decreasing to 0.064 nmol Pi mg^{-1} min^{-1} (54% of control) at 15 μM Al. Vacuolar ATPase activity remained essentially constant from 0 to 10 μM $AlCl_3$. This is an interesting contrast to the Al-induced increase observed in wheat [2,17] and barley [18,19].

To determine the mechanism by which ATPase activities change during Al stress, expression of V-ATPase and F_1F_0-ATPase subunits was examined by Western and Northern analysis (Fig. 3). Levels of the transcript encoding the F_1F_0-ATPase α subunit increased to 201% of control levels by 7.5 μM $AlCl_3$, followed by a decline to 135% by 15 μM (Fig. 3A,B). This pattern closely resembles that observed for increased F_1F_0-ATPase activity (Fig. 2). Protein levels of the F_1F_0-ATPase α and β subunits also increased with increasing $AlCl_3$ concentration (Fig. 3C). Interestingly, levels of these subunits continue to increase past 5–7.5 μM $AlCl_3$, the peak concentrations for F_1F_0-ATPase activity and transcript levels.

Levels of the transcript encoding the V-ATPase B subunit remained nearly constant over the range of Al concentrations tested (Fig. 3A,B), with only a slight increase to 123% of control at 5 μM $AlCl_3$. The amount of protein also remained constant (Fig. 3C), reflecting the constant level of V-ATPase activity observed during Al stress (Fig. 2). In wheat, V-ATPase transcript, protein and activity increased in parallel with increasing concentrations of Al ([2], C.A. Hamilton, unpublished data).

3.2. V-ATPase mutants, but not F_1F_0-ATPase mutants, are hypersensitive to Al

To test the hypothesis that V-ATPase and F_1F_0-ATPase activities are involved in Al resistance, yeast mutants lacking subunits of these enzymes were tested to determine

whether or not they are hypersensitive to Al. The relative sensitivities of V-ATPase mutants, their parent strain and complemented strains to Al were determined by observing their growth in liquid culture. Cultures were grown in SC medium (pH 4.5) since *vma1* and *vma2* mutant strains grew poorly in the LPP [6] normally used for experiments involving Al. In the V-ATPase mutant strains (*vma1*, *vma2*), there was a striking 40% decrease in growth (from 0.85 and 1.09 control OD_{600} to 0.45 and 0.62 OD_{600} at 75 μM $AlCl_3$, respectively) at all Al concentrations tested, while the parent strain (control OD_{600} 1.28) was unaffected (Fig. 4A). The complemented strains (control OD_{600} values of 1.15 and 1.19, respectively) were similarly unaffected by Al. This confirmed that the V-ATPase mutations are responsible for the Al sensitive phenotype of the *vma1* and *vma2* strains.

The relative sensitivities of F_1F_0-ATPase mutants, their parent strain and complemented strains to Al were also measured (Fig. 4B). In contrast to V-ATPase mutants, F_1F_0-ATPase mutants did not show hypersensitivity to Al from 0 to 150 μM. To determine whether higher concentrations were required to observe phenotypic differences, levels of Al up to 400 μM were tested (Fig. 4B, inset). No discernible differences were observed between the five strains.

3.3. Implications for the energy balance hypothesis

Although the responses of plants and yeast to Al are slightly different, yeast is still a valuable model system for hypothesis testing due to its rapid, simple transformation, short life cycle and amenability to manipulation. However, physiological and structural differences between yeast and plants must be considered when analyzing data from this system. For example, Al toxicity in yeast can be overcome by overexpression of a Mg^{2+} transporter [20], presumably due to inhibition of Mg^{2+} transport by Al^{3+}. In contrast, although addition of Mg^{2+} (or other divalent cations such as Ca^{2+}) alleviates Al^{3+} toxicity in wheat [21], reduced Mg^{2+} availability does not cause Al toxicity symptoms [22]. Also, many gene products induced by Al in plants have no protective effect against Al when expressed in yeast [23]. This is likely because many Al-induced proteins are general stress response proteins (induced in response to oxidative stress and heat shock) which do not necessarily have a role in Al resistance.

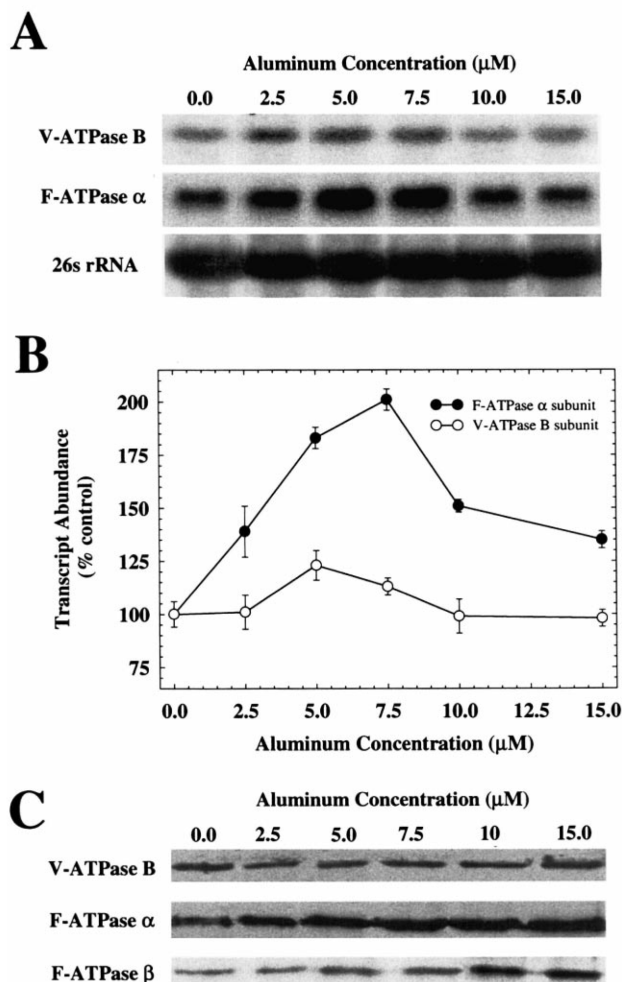

Fig. 3. The effect of Al on expression of V-ATPase and F_1F_0-ATPase. A: Levels of the transcripts encoding the F_1F_0-ATPase α subunit and V-ATPase B subunit were measured by Northern analysis. B: Relative abundance of transcripts as measured by densitometry. The ratio of mRNA/rRNA was measured at each concentration of $AlCl_3$ and compared to the control values for each transcript. C: Determination of protein levels of V-ATPase (B subunit) and F_1F_0-ATPase (α and β subunits) by Western analysis. Cross-reacting bands were visualized using a chemiluminescent detection system. Samples shown are representative lanes selected from triplicates compiled using Adobe Photoshop® v 5.5. Values shown are means ± S.E.M. (n = 3) and are representative of two independent trials.

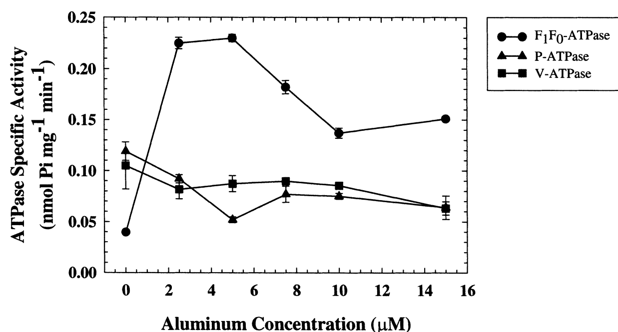

Fig. 2. The effect of Al on cellular ATPase activities (F_1F_0-ATPase, V-ATPase, P-ATPase) in wild-type yeast. Values are means ± S.E.M. (n = 3). Results shown are representative of three independent trials.

It was recently shown by Ezaki et al. [24] that yeast cells exposed to Al show irregular vacuolar morphology. The authors did not speculate as to the possible cause of this effect, but it is possible that Al associated with the yeast vacuole causes this phenotype. Al uptake has been demonstrated in yeast [23,25] and the giant algae *Chara corallina* [26], where it is associated mainly with the cell wall, but is also taken up into the vacuole. Ezaki et al. [23] suggested that intracellular Al does not associate with

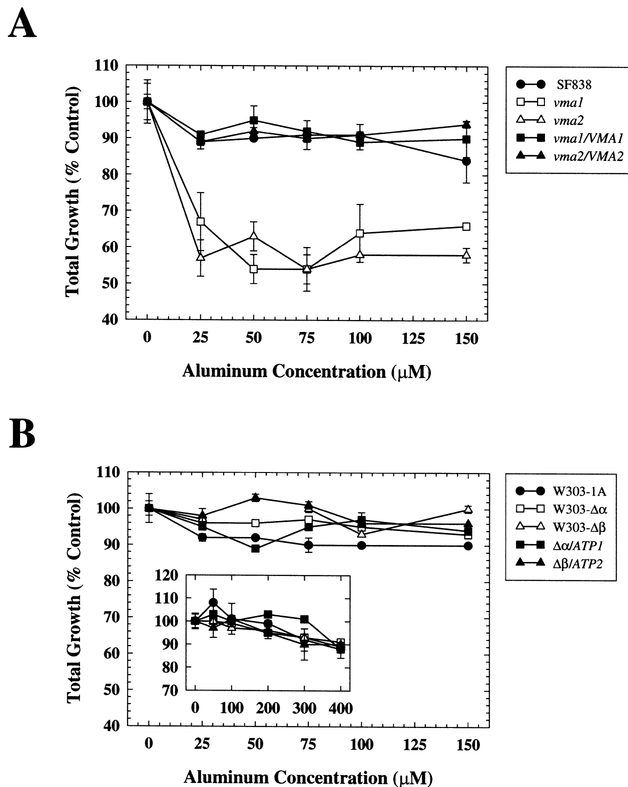

Fig. 4. The effect of ATPase mutations on Al resistance in yeast. A: Yeast strains carrying *LEU2* insertion mutations in the *VMA1* and *VMA2* genes (*vma1*, *vma2*), their isogenic parent strain (SF838), and strains carrying wild-type copies of the *VMA1* and *VMA2* genes on low copy plasmids (*vma1*/*VMA1*, *vma2*/*VMA2*) were grown for 16–24 h in SC minimal medium (pH 4.5) containing 0–150 μM $AlCl_3$. B: Yeast strains with null deletion mutations in the *atp1* and *atp2* genes (W303-Δα, W303-Δβ), their isogenic wild-type parent strain (W303-1A) and strains carrying wild-type copies of the *atp1* and *atp2* genes on low copy plasmids (Δα/*ATP1*, Δβ/*ATP2*) were grown for 16–24 h in SC minimal medium (pH 4.5) containing 0–150 μM $AlCl_3$ (the inset shows that similar results were observed up to 400 μM $AlCl_3$). Total growth (OD_{600}) of each culture was measured and compared to the control (0 $AlCl_3$) values for each strain. Values shown are means ± S.E.M. ($n = 3$). Results shown are representative of three independent trials.

the yeast vacuole based on observations of morin fluorescence, but low levels such as those measured in *Chara* [26] would not be detected using this method. It is possible that Al is sequestered in the vacuole by an active transport mechanism which relies on the tonoplast electrochemical potential. Vacuolar sequestration is thought to be involved in resistance of yeast to a wide range of toxic metals [27,28]. Once in the vacuole, Al could be effectively sequestered by complexing with polyphosphate (as is calcium) [29] or organic acid anions.

Here we have used the yeast model system for simple and direct testing of a hypothesis derived from higher plants. Based on experiments in plants, we hypothesized that the V-ATPase is involved in Al resistance, with the ATP required for its activity supplied by increased activity of ATP synthase. Although V-ATPase transcript and protein levels, as well as enzyme activity, are not affected by increasing Al concentrations in yeast as they are in plants, the observation that V-ATPase mutants are hypersensitive to Al supports our hypothesis. In wild-type yeast, F_1F_0-ATPase transcript, protein and activity levels all increase in response to Al, while F_1F_0-ATPase mutants do not show an Al hypersensitive phenotype. It is possible that the demand for ATP can be met by fermentation, although ATP synthase would normally fulfill this requirement in wild-type yeast and plants. Experiments are underway to test our hypothesis in the model plant, *Arabidopsis*.

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