A *SACCHAROMYCES CEREVISIAE* WINE YEAST STRAIN OVERPRODUCING MANNOPROTEINS SELECTED THROUGH CLASSICAL GENETIC METHODS

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Abstract

Aims: Developing, by classical genetic methods, new wine yeast strains showing improved release of mannoproteins during wine fermentation, as well as suitable selection procedures for this purpose. These strains would be useful to improve quality characters associated to wine mannoprotein content.

Methods and results: UV mutagenesis was used for genetic improvement of the industrial wine yeast strain ADY1. Cell wall-related phenotypes were used as primary selection criteria ; an additional screening procedure was developed based on the detection of the released mannoproteins by hybridization with peroxidase-labeled Concanavalin A. Mannoprotein overproduction was assessed in laboratory media as well as in grapevine juice. One mutant strain, renamed HPS, was selected using these criteria. HPS showed increased mannoprotein release in different culture media, including natural must. Moreover, white wines fermented with this improved strain were less susceptible to protein haze than equivalent wines fermented with the original ADY1 strain. Red wines fermented with the mutant strain were also polysaccharide-enriched as compared to the original one.

Conclusion: No clear correlation between a specific cell wall-related phenotype, or a combination of them, and improved release of polysaccharides by yeast random mutants could be established, and not all strains identified by in vitro assays as mannoprotein overproducing mutants were found positive for mannoprotein release in industrial conditions. Nevertheless, UV mutagenesis, combined with Concanavalin A detection, seems to be a viable way to improve mannoprotein release by industrial wine yeast strains.

Significance and impact of the study: This study is one of the few recent reports on genetic improvement of wine yeast strains by non-recombinant genetic tools. It shows that mannoprotein release can be genetically improved and, for the first time, describes a successful selection procedure for such a complex character. These strains are potentially useful for the improvement of mannoprotein-related characters of white and red wines.

Key words: yeast mannoprotein, *Saccharomyces cerevisiae*, genetic improvement, protein haze, color

Résumé

Objectif: Développer par des méthodes de génétique classique de nouvelles souches de levure montrant une meilleure libération des mannoprotéines pendant la fermentation ainsi que les méthodes de sélections de ces nouvelles levures. L'intérêt de ces souches est d'améliorer la qualité sensorielle des vins, associée à leur teneur en mannoprotéines.

Méthodes et résultats: L'amélioration génétique a été réalisée par mutagenèse UV. Le premier critère de sélection a été la recherche de plusieurs phénotypes en relation avec la paroi cellulaire ; la libération des mannoprotéines a ensuite été détectée par hybridation avec la Concanavalin A. La surproduction des mannoprotéines a été étudiée sur des milieux de laboratoire et sur jus de raisin. Une levure, dérivée d'une souche industrielle, et sélectionnée selon ces critères, a montré une surproduction de mannoprotéines sur plusieurs milieux y compris sur moût naturel. Les vins blancs fermentés par cette souche sont moins sensibles à la casse protéique, que des vins fermentés par la souche originale. Les vins rouges produits à l'échelle industrielle sont aussi enrichis en mannoprotéines comparés à la souche parentale.

Conclusion: Il n'y a pas un phénotype unique en relation avec la paroi qui soit « tout seul » corrélé avec la libération des mannoprotéines. Pas toutes les souches identifiées *in vitro* comme surproductrice de mannoprotéines le sont après production industrielle. La mutagenèse à l'UV combinée avec la détection par Concanavalin A paraît donc être une voie viable pour améliorer la libération des mannoprotéines par des levures œnologiques.

Signification et impact de l'étude: Cette étude fait partie des récentes techniques d'amélioration génétique des souches de levure œnologiques par des méthodes non-recombinantes. Elle montre que la libération des mannoprotéines peut être améliorée génétiquement, et pour la première fois décrit un procédé de sélection pour ce phénotype complexe. Ces souches sont particulièrement intéressantes pour l'amélioration des caractères organoleptiques des vins rouges et blancs.

Mots clés: mannoprotéines de levure, *Saccharomyces cerevisiae*, amélioration génétique, casse protéique, couleur

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INTRODUCTION

Saccharomyces cerevisiae cell wall consists of two layers : an inner layer made of β-1,3-glucan and chitin, and an outer layer consisting of β-1,6-glucan and mannoproteins. Mannoproteins are highly glycosylated proteins whose carbohydrate fraction is constituted by around 98 % mannose and 2 % glucose. Most of them are covalently linked to the inner cell wall layer, either directly to the β-1,3-glucan matrix or indirectly via a β-1,6-glucan branch (Klis *et al.,* 2002).

During the winemaking process, apart from products and by-products of sugar metabolism, yeast cells release cell constituents, like proteins and polysaccharides, which contribute to wine quality. Special attention has been paid during the past 15 years to mannoproteins, involved in several positive quality and technological traits of wines (Caridi, 2006). This may include protection against protein and tartaric instability (Ledoux *et al.,* 1992 ; Feuillat *et al.,* 1998) ; retention of aroma compounds (Lubbers *et al.,* 1994 ; Wolz *et al.,* 2005) ; reduction of astringency (Escot *et al.,* 2001) ; and increased body and mouthfeel (Lubbers *et al.,* 1994 ; Saucier *et al.,* 1999 ; Vidal *et al.,* 2004), especially appreciated in red wines. Finally, mannoproteins seem to stimulate the growth of lactic acid bacteria (Guilloux-Benattier *et al.,* 1995 ; Rosi *et al.,* 1999), and consequently malolactic fermentation, and to improve the foam quality of sparkling wines (Feuillat, 1987 ; 2003).

Considering all that, there is great interest in the isolation and/or development of industrial wine yeast strains able to secrete higher amounts of mannoproteins during the course of wine fermentation than the strains currently available. However, mannoprotein release is a complex character that is difficult to use as a selection criterion, especially for the screening of a large number of strains. For that reason, our group has been working during the past few years on the identification of genetic determinants of mannoprotein release. We have previously shown that mutations in some specific genes can result in increased release of mannoproteins during alcoholic fermentation (Gonzalez-Ramos and Gonzalez, 2006). Some of these mutants were affected in genes related to cell wall biosynthesis or showed weakened cell wall structures. We have also constructed recombinant wine yeast strains improved for mannoprotein release by targeted gene deletion of these genes (Gonzalez-Ramos *et al.,* 2008 ; 2009). However, recombinant strains would face many limitations if they were to be introduced in the market. It would be much more interesting to use classical genetic methods to improve the mannoprotein release character. In the present work, we have developed a screening procedure based on cell wall-related phenotypes in order to identify UV-induced random mutants from a commercial wine yeast strain. These mutants overproduce mannoproteins in laboratory and natural media. Specific tests for the release of mannoproteins in plate assays have also been developed.

MATERIALS AND METHODS

1. UV mutagenesis and strain characterization

S. cerevisiae Active Dry Yeast 1 (ADY1), a commercial wine yeast strain provided by Lallemand Inc., was used in the mutagenesis experiments. Vegetative cells from an overnight culture grown in YPD (1 % yeast extract, 2 % peptone and 2 % glucose) at 30 ºC and 150 rpm were plated on YPD agar (YPD + 2 % agar) to give approximately 500 cells per plate (total of 10 plates). Plates were irradiated with a G8T5 germicidal UV tube to a survival rate of about 50 %, placed immediately in the dark for at least 2 hours and incubated for 48-72 hours at 20 ºC. Around 1700 colonies were recovered from the UV mutagenesis assays (all the isolated colonies were used to avoid any bias) and re-streaked on YPD master plates at 100 strains per plate.

These colonies were replica plated in different media in order to examine their phenotypes. Temperature and caffeine sensitive autolysis phenotypes were screened based on the release of active alkaline phosphatase to the medium (Cabib and Duran, 1975 ; Molero *et al.,* 1993). Colonies were replica plated on YPD supplemented with BCIP (5-bromo-4-chloro-3-indolyl phosphate, 40 mg/L), incubated at 20 ºC for 24 hours and transferred to restrictive temperature (37 ºC). Autolytic mutant colonies stained green-blue after 18-24 hours at 37 ºC. The caffeine sensitive colonies were detected on YPD supplemented with BCIP (40 mg/L) and caffeine (12 mm). Resistance to Congo Red was screened by replica plating the colonies on YPD supplemented with Congo Red (4 or 8 mg/ml). Tolerance to copper was screened on YPD plates containing copper sulfate (2 mM). Media components were purchased from ADSA Micro (Spain) and all other reagents were purchased from Sigma-Aldrich (Spain).

2. Detection of mannoproteins

Mannoproteins were detected in two different ways : (1) in solid medium and (2) in the supernatant of GCY liquid medium (2 % glucose, 2 % Casaminoacids (BD, Sparks, MD, USA), 0.67 % Yeast Nitrogen Base (BD)) or synthetic must (20 % glucose, 0.6 % malic acid, 0.3 % tartaric acid, 0.03 % citric acid, 0.67 % Yeast Nitrogen Base (BD) pH 3.5). For the screening in solid medium, different dilutions of exponential phase cultures were spotted on minimal medium plates (2 % glucose, 0.67 % Yeast Nitrogen Base, 1.67 % agar). Colonies on these plates were covered with a sterilization membrane (pore size, 0.45 µm) and a Hybond-N nitrocellulose membrane placed on top of it. A diagram of the transfer set up is

shown in figure 1. The membrane (A) was incubated for 8 hours at 20 ºC and after drying at 37 ºC, mannoproteins were detected by hybridization with peroxidase-labeled Concanavalin-A (Gonzalez-Ramos and Gonzalez, 2006). For the detection of mannoproteins in liquid medium, the strains were grown in GCY or synthetic must medium. Polysaccharides in the supernatant were quantified and mannoproteins were detected as previously described (Gonzalez-Ramos and Gonzalez, 2006).

3. Fermentation experiments and protein haze

Fermentation assays of Sauvignon Blanc must were carried out at 25 ºC. Must was inoculated to a cell concentration of 10^6 cells/ml and fermentation was monitored by weight loss. Once the fermentations were completed, the wines were centrifuged to eliminate the cells and the protein stability of five replicates was assessed via the heat-induced protein haze. For the stabilization experiments of a white wine, the mannoproteins released by ADY1 and HPS were purified by size exclusion chromatography and concentrated by freeze-drying. These mannoproteins were subsequently added to a heat unstable white wine to a final concentration of 200 mg/l, and these wines were subjected to a heat test in five replicates. Statistical significance of the results was determined by ANOVA analysis.

4. Industrial scale red wine production

Fermentation was performed in two different tanks with the grape variety Aragonês following the traditional protocol for red wine production in the Alentejo D.O. (Denomination of Origin), Portugal. After malolactic fermentation, wines were filtered and analyzed following the Codex analytical methods (Amerine, 1976): color index (absorbance at 420, 520 and 620 nm); total phenolics (IPT, Folin Ciocalteu Index); total anthocyanins (Ribereau-Gayon *et al.,* 1998); total polysaccharides (Feuillat *et al.*, 1989); and protein stability (Dubourdieu, 1988). These analyses were performed by Excel Iberica S.L. laboratories.

RESULTS

1. Mutagenesis and cell wall-related phenotypic assays

Prior to phenotypic analysis, the assay conditions were optimized by replica plating the control strain (ADY1) and a limited number of putative mutant strains on YPD medium containing varying concentrations of caffeine, Congo Red or copper sulfate. The concentration finally chosen for each inhibitory agent was, for resistance phenotypes, the lowest concentration showing an inhibitory effect on ADY1 growth with some putative mutants showing resistance, and for susceptibility phenotypes, the highest concentration showing no or low inhibitory effect on ADY1 growth with some mutants already showing susceptibility. The final concentrations used for the screening were those mentioned in Materials and Methods. No optimization was needed for the concentration of BCIP, used to highlight cell lysis in temperature and caffeine sensitivity analysis. A total of 326 strains (out of the initial 1700 ones) were selected in the first round of screening, of which only 172 were retained after a second validation round. Of these, 25 already stained green-blue on BCIP-containing plates at 20 ºC and 39 stained blue at 37 ºC, 117 showed caffeine sensitivity (impaired growth and blue staining in the presence of caffeine), 56 were resistant to copper, and 22 and 46 were resistant to Congo Red at 4 and 8 mg/ml, respectively. Fifty-two strains were positive for two or more phenotypic criteria.

2. Release of mannoproteins in laboratory media

A more specific screening for the release of mannoproteins was performed on the selected 172 strains, using the experimental setup described in Material and Methods. This screening was designed to detect the mannoproteins released from the yeast cells, and recovered on nitrocellulose membranes, by peroxidase-labeled Concanavalin A detection. The final setup was the result of several trials in order to avoid the background derived from the culture medium and from whole cells attaching to the nitrocellulose membranes. This was accomplished by substituting the yeast extract-containing YPD medium

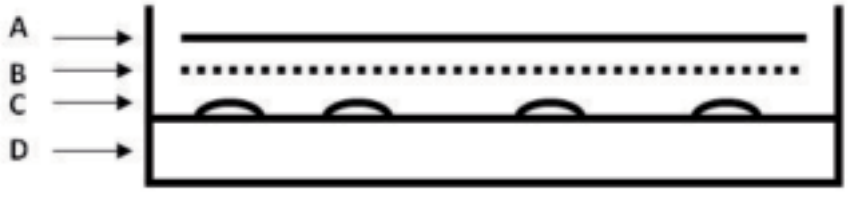

Figure 1 - Diagram of the transfer set up for the detection of mannoproteins released from yeast colonies. A, nitrocellulose membrane disc; B, sterilization filter disc; C, yeast colonies; D, agar medium in Petri dish.

for a minimal medium and by placing a sterilization membrane between the yeast colonies and the nitrocellulose membrane (figure 1). Twenty-two strains showing stronger signals than the control strain were identified as having a potentially higher mannoprotein release than the original strain. The phenotypes of these positive strains in the primary screening are shown in table 1. These are diverse and include both strains positive for one of the selection criteria and strains positive for two or three of them. None of these criteria could thus be selected at this stage as having the highest predictive value for mannoprotein release. Indeed, most of the strains tested in the polysaccharide release assay in liquid medium (GCY) showed a phenotype similar to the control strain (data not shown). Consequently, only 4 strains (5.50, 10.50, 17.9 and 17.49) were selected for further analysis. The amount of polysaccharides released by each of these strains is shown in figure 2.

These four strains were used in fermentation experiments of synthetic must. Sugar consumption and polysaccharide release were monitored along the fermentation process. Fermentation kinetics in synthetic must was similar for all the strains tested, with the exception of strain 17.9, which was slightly impaired (figure 3). Only strain 17.49 clearly released increased amounts of polysaccharides along the fermentation process (up to 30 % higher), as compared to the original strain (figure 3). The strain 10.50 showed similar levels to 17.49 at the last time point but not for the samples taken during active alcoholic fermentation, so we decided to focus on strain 17.49. In order to confirm that the 17.49 strain was actually a derivative of ADY1, they were both compared for mitochondrial RFLP (Legras and Karst, 2003) and for inter-delta element amplification patterns (Querol *et al.*, 1992). No differences were found between both strains (data not shown), thus confirming that strain 17.49 is a derivative of ADY1. Strain 17.49 was then retained for further analysis and renamed HPS.

3. Stabilization of wines with mannoproteins released by HPS

Mannoproteins produced by ADY1 or HPS during the fermentation of a synthetic must were recovered as described in Materials and Methods and used for the

Table 1 - Phenotypes in the primary screening of the strains showing the strongest signals in the mannoprotein release assay in solid medium.

	Phenotype in the primary screening				
Strain	Autolytic (BCIP) 20ºC	Autolytic (BCIP) 37ºC	Caffeine sensitive	Copper resistant	Congo Red resistant
3 101	+	+	+		
5.50	+	+	+		
9.54	+	+			+
10.4	+	+	+		
10.50			+		
10 101			+		
11.4			+		
11.85				+	
12.2					+
15.12				+	+
15.59		+			
15.87		+			
15.94			+		
16.13	+				
16.16			+		
16.45		+	+		
16.53				+	+
16.82			+		
16.90	+	+	+		
17.9			+		
17.49		+		+	+
17.59			+	+	+

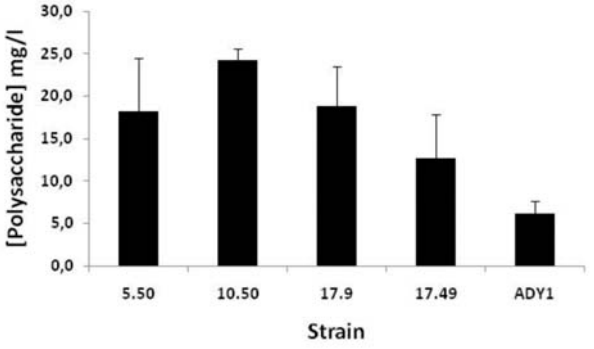

Figure 2 - Polysaccharides released in GCY liquid medium by four selected strains and the original ADY1 strain.

Table 2 - Average turbidity (nephelos) in the heat test of three Sauvignon blanc wines fermented with the original ADY1 and the improved HPS strain, including the p value of the ANOVA comparison between both strains.

Fermentation number	HPS	ADY1	p value
1	514.2	602.25	0.00459
2	236	351.25	8.56*10-6
3	396	473	0.02

stabilization of a heat unstable white wine. In the heat test, the turbidity of the wine treated with material from ADY1 was not statistically different from the untreated wine, while the turbidity of the wine treated with material from HPS was significantly lower ($p < 0.05$) than with ADY1 material (mean values of 124 and 177 nephelos turbidity units (NTU), respectively) (data not shown).

4. Protein haze stability of wines fermented by HPS

Stabilization of white wines against protein haze is one of the first beneficial properties associated to yeast mannoproteins. We chose this technological property to confirm the positive results obtained with the strain HPS. Three independent fermentation experiments of must from a white grape variety with high protein content (Sauvignon Blanc) were carried out with the strains HPS and ADY1. Under these experimental conditions, the fermentations were completed and the time course of fermentations was similar with both strains (data not shown). The turbidity of the wines fermented with strain HPS ranged from 236 to 514.2 NTU corresponding to values of 67 to 85 % that of ADY1 (table 2). This result is in accordance with the results from synthetic musts discussed above. Based on the reported effect of yeast cell wall mannoproteins on protein haze formation in white wines (Ledoux *et al.*, 1992; Waters *et al.*, 1994; Brown *et al.*, 2007; Schmidt *et al.*, 2009; Lomolino and Curione, 2007; Dupin *et al.*, 2000), the most plausible hypothesis is that the increased amounts of polysaccharides released by the HPS strain are responsible for the increased protein stability of wines fermented with this strain.

5. Stability of the « protein haze stabilization » trait after industrial active dry yeast production

Industrial wine yeast strains are usually sold as active dry yeast and its production process is highly stressful for yeast cells. In addition, it has the potential to induce genetic instability. It was therefore important to test whether the active dry yeast production process had any effect on the stability of the improved « protein haze stabilization » trait of HPS. The strain was submitted to a pilot scale active dry yeast production process, and was conditioned in the same way as the original ADY1 strain. Laboratory fermentations were then performed with Sauvignon blanc musts and the HPS and ADY1 strains obtained from commercial preparations. Protein haze stability assays were again performed with the clarified wines and indicated that the stabilization trait has been maintained throughout the industrial production of the active dry HPS strain (data not shown).

6. Industrial scale red wine fermentation

Red wines were produced (D.O. of Alentejo, Portugal) and analyzed as described in Materials and Methods, using the HPS mutant and the original ADY1 strain. The results are shown in table 3. The main parameter to be considered in this industrial trial was the polysaccharide content of the wines, which would confirm the increased potential of HPS to release polysaccharides during the wine production process. This was indeed confirmed

Table 3 - Analytical data of Aragonês wines fermented with either the HPS improved strain or the original ADY1 strain.

	Total polysaccharides (mg/l)	Total anthocyanins (mg/l)	Color Index (DO 420+520+620 nm)	IPT (DO 280 nm)
ADY1	520±30	627	9,92	65,2
HPS	615±26	644	10,54	66,3

since the samples from wines fermented with HPS yeasts contained about 100 mg/L more polysaccharides than those fermented with ADY1. The color and polyphenol content values suggest that this increased polysaccharide content is positively influencing the color stability of the HPS wines, but further confirmation is needed.

DISCUSSION

In previous works (Gonzalez-Ramos and Gonzalez, 2006 ; Gonzalez-Ramos *et al.*, 2008 ; 2009) we have shown that all the mutations leading to increased mannoprotein release in both laboratory and wine yeast strains were recessive. The mannoprotein release phenotype was only detected in haploid or homozygous deletion strains. This was not surprising since all the mutations tested in these works were indeed complete ORF deletions of the target gene. Anyhow, this would constitute a limitation for the genetic improvement of industrial wine yeast strains by random mutagenesis of vegetative cells, as done in this work (ADY1 ploidy is around 2n), because almost all mutant strains are expected to be heterozygous, and the probabilities of finding a strain carrying a mutation in at least two alleles of a given gene is very low. In this condition, mutations leading to the desired phenotype would be rare, probably as a result of either the loss-of-function of genes whose alternative allele is already non functional, or the low frequency of semi-dominant or dominant mutations. This would explain why only one out of the 1700 primary isolates could finally be confirmed as a mannoprotein overproducing strain in high sugar content media. The production of potentially useful strains would probably increase by using haploid derivatives of wine yeast strains (whenever available), mass segregation of spores derived from mutagenized cells, or mutagenesis of spores. However, the possible effect of the segregation process on the enological characters of the yeast strains must also be considered before choosing one of these alternative strategies.

It is worth noting the different behavior of the yeast strains in laboratory media and in synthetic and natural musts. Among the four strains that showed increased mannoprotein release in GCY medium (figure 2), only strain 17.49 (HPS) showed a clear increase in mannoprotein release in synthetic must (figure 3). Curiously, this strain was the less promising among the four strains shown in figure 2. In addition, the mannoprotein overproduction level for HPS decreased from about 100 % in GCY medium to just around 30 % in synthetic must. The overproduction level seems to be more consistent between synthetic and natural must fermentations. This moderate increase in mannoprotein release during the fermentation process seems to be enough to influence mannoprotein-related properties of the wines. This is clearly shown in table 2 for protein haze, and seems to be suggested for red wine fermentations (table 3). Additional studies are ongoing in order to confirm the impact of this increased mannoprotein content on yeast polysaccharides in the color stability of wines after a few months of aging in bottle.

CONCLUSION

In conclusion, we have shown UV mutagenesis to be an appropriate tool to generate non-recombinant yeast strains exhibiting an improved release of polysaccharides during the course of wine fermentations. The use of cell wall-related phenotypes as primary selection criteria has shown to be successful ; however, no clear correlation between a specific cell wall-related phenotype, or a combination of them, and improved release of polysaccharides could be established. The strain HPS has shown to be genetically stable and seems to be a good candidate for industrial dry yeast production. This strain could eventually be useful for the improvement of other wine properties for which a positive effect of yeast polysaccharides and/or mannoproteins has been shown.

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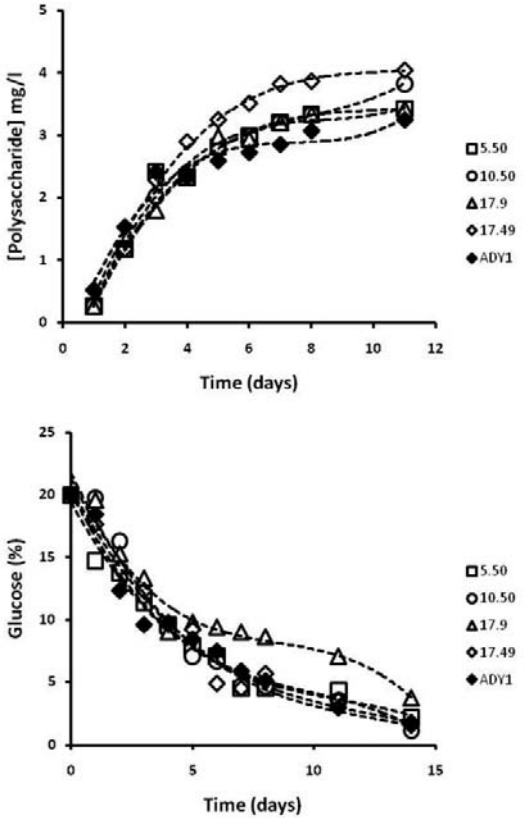

Figure 3 - Fermentation of a synthetic must by the mutant strains and the original ADY1 strain.
Upper panel, concentration of released polysaccharides. Lower panel, glucose consumption.

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