

Expression of Zinc Finger Protein Zat12 from *Arabidopsis thaliana* in *Escherichia coli*

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Abstract

The C2H2 zinc finger protein ZAT12 has been classified as a plant core abiotic stress response gene in the early response to multiple stresses. ZAT12 links the iron deficiency and oxidative stress responses through the direct interaction with/and negative regulation of a central regulator - FIT. For further research on the regulation of the ZAT12 protein *in planta*, a huge quantity of ZAT12 proteins is required to inject into mice for the generation of ZAT12 antiserum. In this study, the gene encoding the ZAT12 protein from *Arabidopsis thaliana* was cloned into the expression vector - pETBlue-2 and then overexpressed in *E. coli* T7. A high expression level was indicated by SDS-PAGE. Immunoblot demonstrated successful expression using a bacterial expression system.

Keywords

ZAT12, zinc finger protein, ZAT12 expression

Introduction

The zinc finger of *Arabidopsis thaliana* 12 (ZAT12), a member of the C2H2-type plant-specific zinc (Zn) finger transcription factor family (Englbrecht *et al.*, 2004; Ciftci-Yilmaz and Mittler, 2008; Miller *et al.*, 2008; Kielbowicz-Matuk, 2012), contains an EAR motif and is thought to function as a repressor of gene expression (Kagale *et al.*, 2010). ZAT12 was identified among genes induced by light (Iida *et al.*, 2000; Davletova *et al.*, 2005b), low temperatures (Fowler & Thomashow, 2002; Kreps *et al.*, 2002; Vogel *et al.*, 2005), wounding (Cheong *et al.*, 2002), osmotic and salinity stress (Kreps *et al.*, 2002), and oxidative stress (Rizhsky *et al.*, 2004; Davletova *et al.*, 2005b; Vanderauwera *et al.*, 2005). ZAT12 is also the direct target of many transcriptional regulators such as EIN3, bZIP29, AtUSB1, DRM2, At3g03170, and LEA18 (Peng *et al.*, 2014; Ben Daniel *et al.*, 2016). However, the role of the ZAT12 protein in the abiotic stress signaling network has not been fully elucidated.

Recently, ZAT12 was identified in an attempt to find a link between iron deficiency responses and oxidative stress. In conditions

Received: October 26, 2019
Accepted: June 1, 2020

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where iron is poorly available, plants are able to induce a set of genes that enhance the efficiency of its uptake by the roots. A central regulator of this response in the model plant *Arabidopsis* (*Arabidopsis thaliana*) is the basic helix-loop-helix transcription factor FIT whose activity is tightly regulated. Using a yeast two-hybrid assay and BiFC, the EAR motif was demonstrated to be necessary for the interaction between FIT and ZAT12 (Le *et al.*, 2016). The expression of the *FIT* gene was upregulated in *zat12* loss-of-function plants. In addition, these plants accumulated a higher amount of iron compared to the wild type. ZAT12-GFP fluorescence was detected in *Arabidopsis* roots, where it could be observed in the nuclei. This protein was elevated upon iron deficiency, H₂O₂, and MG132 treatments. ZAT12 is considered as a link between iron deficiency and oxidative stress responses through direct interaction with and negative regulation of FIT. These functions of the ZAT12 protein were only demonstrated using a ZAT12-GFP protein with a GFP antibody in immunoblot experiments (Le *et al.*, 2016). To verify this native ZAT12 function, i.e. to monitor Fe dependent expression and the regulation of ZAT12 protein in planta, it is necessary to generate an anti ZAT12 antibody. To do this, we have conducted a series of experiments including the cloning, transformation, heterologous expression, and purification of the recombinant protein. In this study, the ZAT12 recombinant protein was successfully expressed in *E. coli*.

Materials and Methods

Strains, expression vector, and culture conditions for cloning

E. coli Nova Blue cells were used as hosts for transformation. *E. coli* strains carrying the pETBlue-2 vector (**Figure 1**) were grown in Luria-Bertani medium supplemented with 100µg of tetracycline/mL when required at 37°C. The DNA fragments for subcloning were isolated from agarose gels and cloned into the pETBlue-2 vector using a Perfectly Blunt® Cloning Kit. (Novagen, Germany). Tuner (DE3) pLacI *E. coli* host strains that are compatible with the pET

expression systems from Novagen were used for the expression of the ZAT12 protein. Other standard microbial and recombinant techniques used throughout this work were as described by Sambrook *et al.* (2001).

Generation of the ZAT12-His gene constructs using PCR

The coding sequence of ZAT12 from *Arabidopsis thaliana* was amplified by PCR using the primer combination 5' ZAT12_PET (5'-ATGGTTGCGATATCGGAGATCAA-3') (**Figure 2**) and ZAT12_CT His 3' (5'-TCAAGAGGCCATACCGTGATGATGATGATGATGATGAGAACCACGATAAACTGTTCTTCCAAGCTCCA-3') (Le *et al.*, 2016). After checking the size of these fragments by agarose electrophoresis, the *ZAT12-His* fragments were purified and cloned into the EcoRV cloning site of the pETBlue-2 vector using the Perfectly Blunt® Cloning Kit.

Transformation

The recombinant plasmid was transformed into NovaBlue Singles™ Competent Cells (Novagen, USA). Identification of successful cloning events was performed by blue/white screening. The insertion of *ZAT12-His* into the pETBlue-2 vector leads to the disruption of the expression of the *lacZ* α-peptide, and thereby produces white colonies on plates supplemented with X-gal and IPTG (Isopropyl-β-D_thiogalactoside from Roth, Karlsruhe, Germany). Inversely, colonies containing the vector without insertion turn blue. After the initial selection of positive colonies, colony PCR and sequencing were performed for verification of positive recombinant plasmids. After that, the recombinant plasmid was transformed into Tuner (DE3) pLacI cells and the recombinant protein induction was performed according to the manufacturer's instructions (Novagen, USA).

Protein electrophoresis and Immuno Blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular mass of the protein using 10% gels (Laemmli *et al.*, 1970).

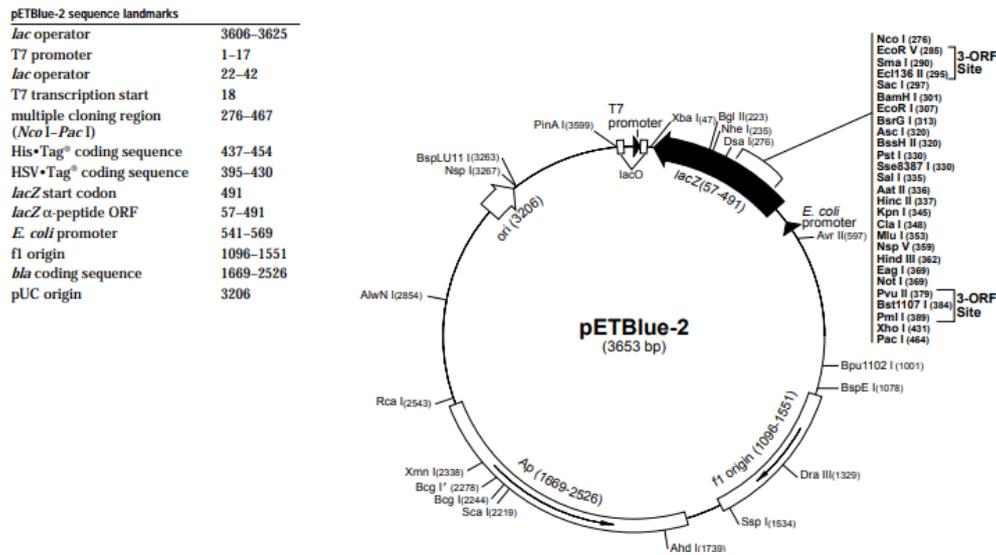


Figure 1. Map of the pETBlue-2-vector. Overview of the pETBlue-2-vector map with multiple cloning sites (Novagen, Germany)

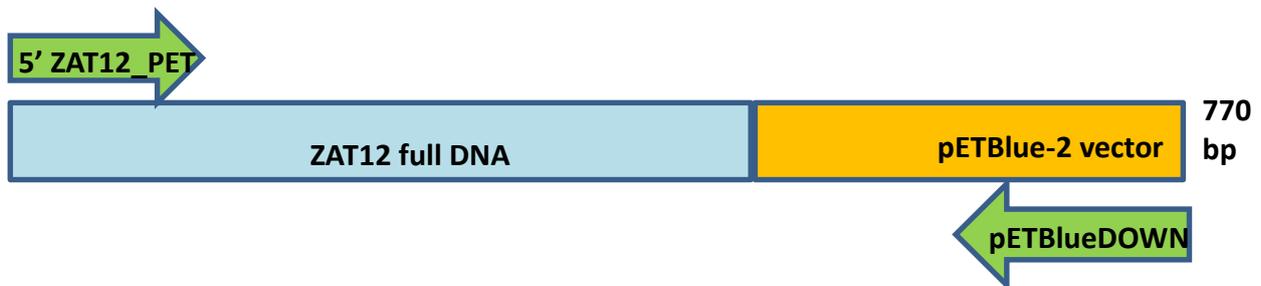


Figure 2. Schematic view of the amplified fragment for cloning and colony PCR. A. The ZAT12 full DNA sequence was amplified using the 5'ZAT12_PET and ZAT12_CT His 3' primers. The 6x His Tag was added to the reverse primer. The size of the amplified fragment indicated the length of the ZAT12 full DNA with 6x His sequence. B. The size of the colony PCR product was amplified using the 5' ZAT12_PET and pETBlue Down primers (from Novagen) after transformation.

The bacterial extract containing the recombinant ZAT12 fusion protein was loaded on a preparatory gel and blotted to a nitrocellulose membrane. After Ponceau S staining, the membrane region containing the ZAT12 antigen was cut off as a strip. The membrane was blocked for 1 hour at room temperature with 1% bovine serum albumin (BSA) dissolved in PBS-T and subsequently probed with crude mouse antiserum at 4°C overnight. The unbound fraction was collected into a new tube. The membrane was washed 3 times with PBS-T and the bound antibodies were eluted two times with elution buffer (0.1 M glycine-HCl pH 2.7, 0.5 M NaCl). The eluted antibody fractions were immediately neutralized by adding 1/10 volume of neutralization buffer (1 M Tris-HCl pH 8.0, 1.5 M NaCl, 1 mM

EDTA, 0.5% NaN₃) and BSA was added at a 1 mg mL⁻¹ final concentration (Novagen, according to manufacturer's manual).

For the detection of the ZAT12-His protein, His antibodies and freshly purified undiluted anti-ZAT12 mouse antiserum were applied. These primary antibodies were detected with anti-mouse IgG conjugated with horseradish peroxidase (1:8000 dilution, Sigma-Aldrich, USA).

The ZAT12-His protein was detected by incubation with His rat antibody (1:1000 Roche, Germany) and a secondary antibody anti-rat IgG (whole molecule)-horse radish peroxidase conjugate (1:10000, Sigma-Aldrich, USA). Detection signals were developed using an enhanced chemiluminescence detection kit

(Biorad, USA) according to the manufacturer's protocol.

ZAT12 antibody preparation

Based on the predicted antigenic propensity scores, a peptide corresponding to the N-terminal of ZAT12 was chemically synthesized and conjugated with KLH (Bio Trend) and later injected into mice to obtain a polyclonal antiserum (this work was conducted by Prof. U. Müller, Zoology Department, Saarland University). In this study, the antiserum was tested for its specificity by detecting bacteria positively expressing ZAT12.

Results

Plasmid construction and confirmation of cloned recombinant ZAT12 plasmid

To generate the recombinant plasmid, the full-length DNA sequence of ZAT12 was specifically amplified with the ZAT12-PET and ZAT12-CT-His primers, which produced an expected band of 538bp. Upon successful ligation and transformation, the obtained colonies were numbered and a colony PCR was performed to check the positive clones for the presence of the recombinant plasmid. In addition,

sequencing of the selected recombinant plasmid was performed to confirm the proper orientation of the insert by ligation (**Figure 3**).

Resultant colonies were tested for the presence of the recombinant plasmid by colony PCR, and colonies were numbered as 1, 2, 3,..., 10. If the insert was in the correct orientation, the expected size of the PCR product for ZAT12 with the primer combination (ZAT12 5' and pETBlueDOWN, see **Figure 2**) was approximately 800bp (538bp of ZAT12 full plus 232bp from the pET Blue2 vector). Colony no. 8 of ZAT12 gave a PCR product at the expected size.

Expression of ZAT12 protein

After verifying the sequence, the plasmid was transformed into BL21 (DE3) cells of *E. coli* for expression. SDS-PAGE analysis detected the expected products from BL21 (DE3) cells induced with IPTG. The results are shown in **Figure 4**.

After colony PCR confirmation, the selected recombinant plasmid was transformed into the Tuner™ DE3 expression cells. Upon successful expression of the recombinant 18kDa ZAT12-His fusion protein at a small scale level, a large scale expression of ZAT12 protein was performed.

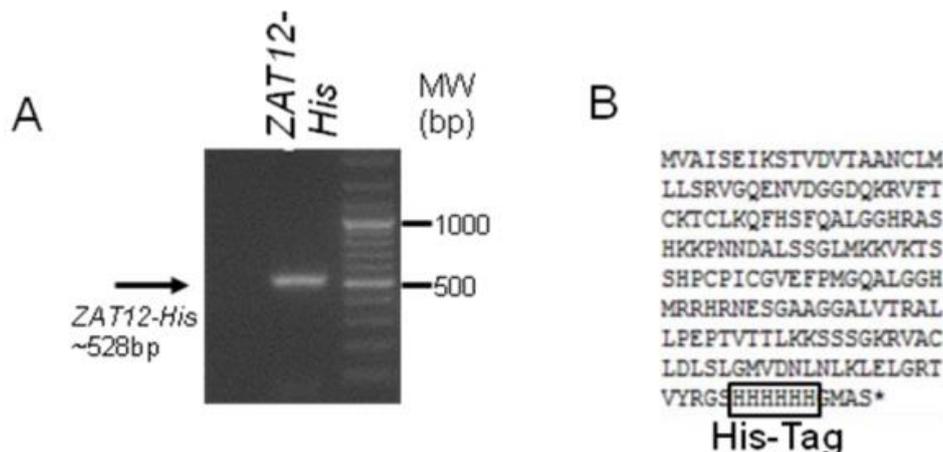


Figure 3. Preparation of the ZAT12 encoding gene. A. The DNA fragment of ZAT12-His using PCR. B. Amino acid changes at the 5'-ends of ZAT12 introduced via PCR. C. Colony-PCR of ZAT12-His colonies was performed on 10 colonies.

Resultant colonies were numbered as 1, 2, 3,..., 10. Asterisks (*) indicate the ~800bp size ZAT12 fusion DNA band (from 538bp of ZAT12 full plus 232bp from the pET Blue2 vector). The only colony no. 8 gave a PCR product at the expected size, Figure 1A. M=ladder. Colony no. 8 was a positive colony.

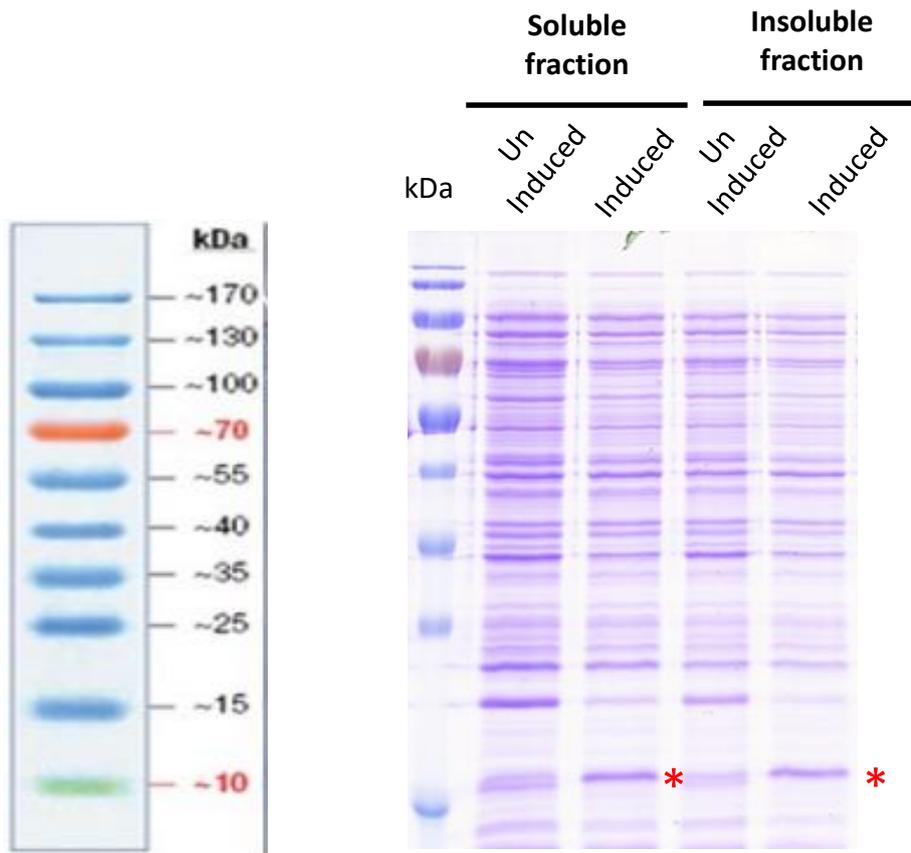


Figure 4. SDS-PAGE analysis of the heterologously expressed recombinant ZAT12 fusion protein in *E. coli*. ZAT12 fusion protein expression in Tuner™ DE3 cells accumulated as insoluble proteins (inclusion bodies) and soluble fraction. Asterisks (*) indicate the position of ~18kDa ZAT12 fusion protein band (from ZAT12 full gene plus 6 His tags).

Specificity test of the ZAT12 protein

The heterologously expressed recombinant ZAT12 was purified for affinity and injected into mice. The immunization of mice and collection of antiserum were kindly performed by Prof. Uli Müller and Iris Fuchs, Department of Zoology, Saarland University. The obtained antiserum was checked for its specificity on *E. coli* expressing the ZAT12 fusion protein (**Figure 5**). We detected a single band on western blot that matched to the specifically expressed and desired ZAT12 protein so that we were able to conclude that the ZAT12 protein was expressed successfully in *E. coli*.

Discussion

The C2H2-type plant-specific zinc finger transcription factor family was defined by the presence of a conserved zinc finger domain, in

which two cysteines and two histidines coordinate with a single zinc atom to form a finger construct consisting of a short beta-hairpin and an alpha helix. At5g59820 was identified among the genes belonging to this family and named ZINC FINGER OF ARABIDOPSIS THALIANA12 (ZAT12). Structural analysis of the ZAT12 protein showed that it constituted of 162 amino acids divided into two C2H2-type zinc finger domains with a 22-amino acid inter-finger region, and a LDLSL core sequence of the EAR motif localized at the C terminus from amino acid 143 (Meissner & Michael, 1997; Englbrecht *et al.*, 2004; Kagale *et al.*, 2010). In plants, the ethylene-responsive element binding factor associated with the Amphiphilic Repression (EAR) motif is a transcriptional regulatory motif found as an active repressor in members of the ERF, C2H2, and AUX/IAA families, among others (Kagale *et al.*, 2010).

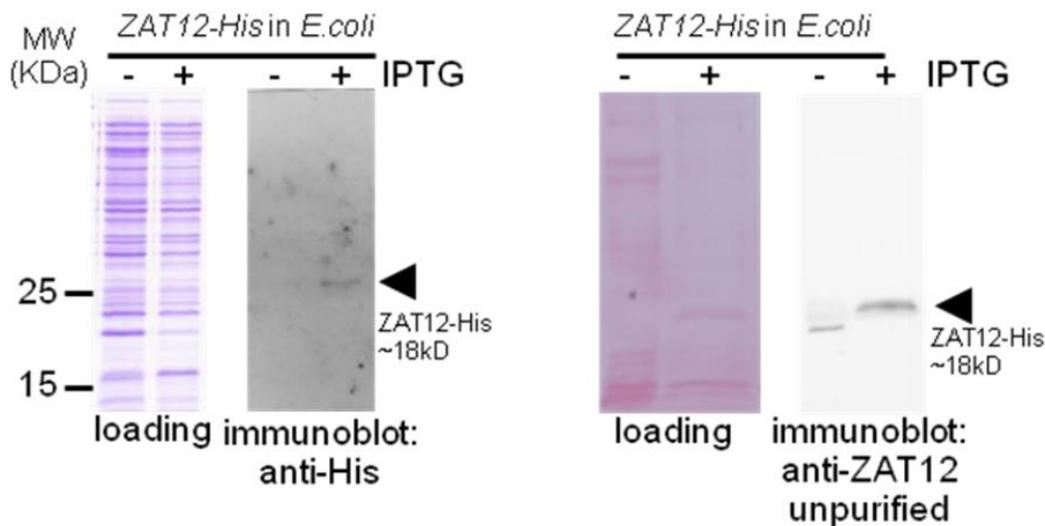


Figure 5. Specificity of the ZAT12 protein. A. The last lane from the right-hand side was loaded with ZAT12 induced bacterial culture and probed/incubated with anti-His antibodies (A), and with anti-ZAT12 unpurified antiserum (B). The triangle (◄) indicates the position of the ~18kDa ZAT12 fusion protein band (from ZAT12 full gene plus 6 His tags).

At5g59820 was identified to be a homolog of the Indica rice ZOS3-22 (Os03g0820400, LOC_Os03g6057, ZFP37). ZOS3-22 expression was also up-regulated in seedlings with a H₂O₂ treatment and in the shoots of plants exposed to Fe deficiency or combined Fe and P deficiency (de Abreu Neto & Frei, 2015).

The coding sequence of ZAT12 from *Arabidopsis thaliana* was amplified using touch-down PCR. The annealing temperature of 58°C was suitable for amplification of the ZAT12 gene. Determination of the optimum annealing temperature for PCR is very important because total genomic DNA extracted from *Arabidopsis thaliana* was used as a template for ZAT12 amplified PCR (Rychlik *et al.*, 1990). The fragment was a band with a size of 538bp, similar to the expected ZAT12 fragment size suggesting successful amplification of *Zat12* from *Arabidopsis thaliana* genomic DNA using ZAT12_PET and ZAT12_CT His primers at the annealing temperature of 58°C.

The transformed Nova Blue bacterial cells were selected using 100 µg mL⁻¹ tetracycline due to the presence of the tetracycline resistance gene in pETBlue-2. Colony PCR on the randomly selected bacterial colonies was conducted using the primers ZAT12 5' and pETBlue DOWN which binds to the vector downstream of the

cloning site to identify transformed bacteria that carry recombinant pETBlue-2 with *Zat12* inserted at the desired orientation. Also, the amplified fragment was approximately 800bp, consisting of 538bp of ZAT12 full and 272bp from the pET Blue2 vector. Lane 8 displayed a band with a size of approximately 800bp (Figure 1C). Furthermore, lanes 1, 2, 3, 4, 5, 6, 7, 9, and 10 were similar in that they showed the absence of a band with a size smaller than 1kb. The results suggested that the colony coded 8 (lane 8) may carry the recombinant pETBlue-2 with *Zat12* insert at the desired orientation. This assumption was tested through isolation of the plasmid from colony coded 8, which was followed by sequencing. The results agreed with the gene map published in the pETBlue-2 Cloning Kits User Manual. Hence, the results suggested that *Zat12* was inserted into the cloning site of pETBlue-2 at the desired orientation. The converted sequence of ZAT12 showed the ZAT12 sequence-tagged 6x His had a band with a size of 1kb (refer to **Figure 3**). The results further confirmed that these plasmid DNA samples were pENTR™/D-TOPO® with *Zat12* inserted in the cloning site at the desired orientation. A high expression level of the ZAT12 protein was indicated by SDS-PAGE and Immunoblot. Using His antibody and ZAT12 antiserum in Immuno Blot to determine the

specificity of ZAT12 showed that ZAT12 was expressed successfully in *E. coli*.

Conclusions

Recombinant ZAT12 was expressed successfully in *E. coli*. This study helped accumulate enough ZAT12 recombinant protein for immunizing/injecting into mice in the following study of ZAT12 antibody generation.

Acknowledgements

The author would like to convey his/her deep thanks to the Laboratory of Botany, Faculty of Biosciences, University, Saarbrücken, Germany for their kind hospitality and support in conducting this study. A special thanks to Professor Dr. Uli Mueller, Department of Zoology, Saarland University, for his kind help in producing anti-ZAT12 antiserum.

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