



Characterization of *crtRB1* Gene Polymorphism and β -Carotene Content in Maize Landraces Originated From North Eastern Himalayan Region (NEHR) of India

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Micronutrient malnutrition or hidden hunger affects a large population in developing and developed countries. One of the major micronutrient deficiencies is vitamin A, which causes several major disorders such as growth retardation, blindness, and increased susceptibility to infectious disease. Maize has been shown for great natural variation for provitamin A carotenoids and it is a promising crop for provitamin A biofortification. *crtRB1* (β -carotene hydroxylase 1) gene is responsible for enhancing β -carotene content in maize. DNA marker linked to the *crtRB1* gene had a considerable effect on enhancing β -carotene content that has been identified previously. In India, the North-East Himalayan region (NEHR) is the center of maize diversity, maize landraces from this area have good agronomic characters and well-adapted to stress environments. However, no prior knowledge about the existence of the *crtRB1* gene and that of the β -carotene content in NEHR maize landraces is available. Hence, the goal of this study was to characterize the *crtRB1* gene polymorphism and β -carotene content in NEHR maize landraces using gene-specific marker (*crtRB1* 3'TE) and high-performance liquid chromatography (HPLC). We screened 26 maize landraces using *crtRB1* 3'TE marker and found that all the maize landraces to be either homozygous to the unfavorable allele or heterozygous (both favorable and unfavorable alleles) but no landraces were homozygous for favorable allele. On the other hand, HPLC analysis showed that the β -carotene content in the heterozygous allele type of landraces varied from 1.36 to 4.40 $\mu\text{g/g}$ while unfavorable homozygous allele type landraces varied from 0.33 to 0.94 $\mu\text{g/g}$. It was found that the landraces CAU-M66 and CAU-M16 possess the highest amount of β -carotene content (4.40 and 4.26 $\mu\text{g/g}$) and had the *crtRB1* favorable allele in heterozygous condition

among the 26 maize landraces studied. This is the first study that disclosed the *crtRB1* gene polymorphism and the β -carotene content in NEHR maize landraces. The newly identified β -carotene-rich maize landraces can be effectively utilized in the breeding programs to enhance the β -carotene content in maize.

Keywords: maize, β -carotene, *crtRB1*, North-East Himalayan Region, vitamin A

INTRODUCTION

Maize (*Zea mays*) is one of the primary cereals in the world and a major contributor to food security in Asia, Africa, and Latin America. It can also be grown in a variety of agro-climatic zones and has the potential to be bred to offer productive cultivars that are attractive to farmers, and consumers, in particular for their nutritional properties (Pingali, 2001). Micronutrient malnutrition or hidden hunger is affecting a large population in developing and developed countries. In particular, Vitamin A deficiency causes serious health issues such as growth retardation, blindness, and increased susceptibility to infectious disease to children and pregnant women (Dary et al., 2002; West, 2003). Maize possesses great natural variation for provitamin A carotenoids, thus maize has been targeted for provitamin A biofortification (Pillay et al., 2014). HarvestPlus has combined with CIMMYT and other partner institutions to develop maize varieties that are high yielding, profitable, suitable to consumers, and also rich in Provitamin A carotenoids comprising of α -carotene, β -carotene, and β -cryptoxanthin, which can be metabolically converted to active vitamin A substances in the human body (Bouis and Welch, 2010; Asson-Batres and Rochette-Egly, 2016). So far, the enhancement of Provitamin A is mostly focused on the selection of β -carotene content. Two genes namely, *crtRB1* (β -carotene hydroxylase 1), and *LcyE* (*lycopene epsilon cyclase*) had a considerable effect on enhancing β -carotene content in maize (Harjes et al., 2008; Yan et al., 2010). β -carotene is hydroxylated to β cryptoxanthin by the *crtRB1* gene and was found to be more effective in increasing the β -carotene content than the *LcyE* (Babu et al., 2013). The *crtRB1* gene is present in three allelic forms due to the *crtRB-3' TE* polymorphism that exists within the gene. Allele 1 (543 bp; with-out TE insertion), is identified as a favorable allele for increasing the β -carotene by decreasing transcript expression of the *crtRB1* gene, while allele 2 (296 + 875 bp; with 325 bp TE insertion), and allele 3 (296 + 1,221 + 1,880 bp; with 1,250 bp TE insertion) cause unfavorable effects (Yan et al., 2010). Polymerase chain reaction (PCR)-based co-dominant markers were identified based on these polymorphisms, and these markers useful to enhance the β -carotene content by marker-assisted selection (MAS). Many studies confirmed that *crtRB1-3' TE* polymorphism is associated with effecting a 2- to 10-fold increase in β -carotene content in maize kernels (Muthusamy et al., 2014; Zunjare et al., 2018; Chandran et al., 2019).

Maize is the principal crop ensuring food security, after rice, cultivated in the North-East Himalayan Region (NEHR) of India. Besides, NEHR is the center of maize diversity in India and maize landraces from this area have good agronomic characters and well-adapted to stress environments. Thus, they are still

conserved and exploited by NEHR farmers for various purposes. Tremendous phenotypic diversity and a large variation in kernel color (i.e., white, violet-brown, yellow, orange-yellow, light-red, dark red, brown-black) were observed in the maize landraces of NEHR. CAU-M16 (Kabullamah), also known as finger maize is popular for its aromatic taste with small size cob. Whereas, CAU-M66 (Chujak) is known for its long cob and large round seeds as well as sweet taste. CAU-M60 (Vekla) is a medium-size cob originated from Ukhrul. However, no information is available about the presence of the *crtRB1* gene and the variation of β -carotene content in these landraces. Thus, the objective of this study was to characterize the *crtRB1* gene polymorphism and determine the β -carotene content variations in maize landraces originated in NEHR of India.

MATERIALS AND METHODS

Plant Genetic Materials and Experimental Plot

A total of 26 maize landraces from NEHR of India showing variations in kernel color (i.e., white, violet brown, yellow, orange-yellow, light red, dark red, brown-black etc.) were collected from farmer's field and used for this study (Figure 1, Table 1). Seeds of these landraces were available at the Central Agricultural University, Imphal, India. Twenty-six maize landraces were planted in a randomized complete block design (RCBD) with three replications at experimental farm, Central Agricultural University, Each landrace was planted in two rows (3 m length) at the spacing of 60 and 30 cm between rows and plants, respectively. Recommended agronomic practices were followed and each entry was carefully self-pollinated to avoid any possible contamination. Ears from each entry were harvested separately from individual plants representing three biological replicates and seeds were shelled and stored in dark conditions at 40°C until carotenoid extraction.

Genomic DNA Isolation and PCR Analysis

The genomic DNA was isolated from 3-week old seedlings using the cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). The isolated DNA was tested for its quality and quantity on a 0.8% agarose gel. The *crtRB1* gene-specific marker "*crtRB1*" (65F: ACACCACATGGACAAGTTCG, 62R: ACACTCTGGCCCATGAACAC, 66R: ACAGCAATACAG GGGACCAG) was used to identify the allelic variation of the *crtRB1* gene. The polymerase chain reaction (PCR) was carried out in 10 μ l reaction containing 25 ng/ μ l of maize genomic DNA, 2 mM MgCl₂, 1 mM dNTPs, 2 μ M of each primer, and 1.5 U if *Taq* polymerase. After an initial denaturation for 5 min

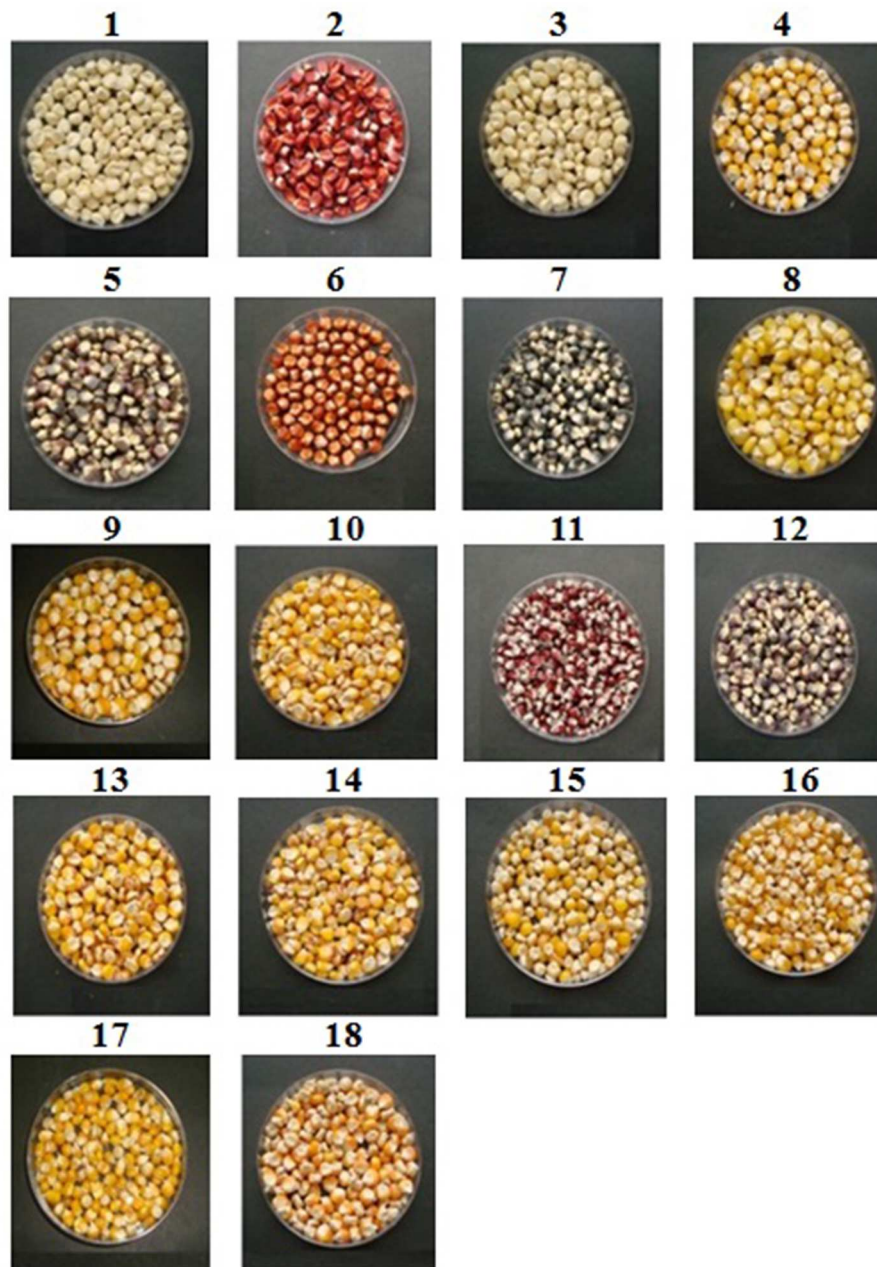


FIGURE 1 | Kernel color variation in NEH maize landraces. (1) CAU-M 1, (2) CAU-M 3, (3) CAU-M 5, (4) CAU-M 12, (5) CAU-M 13, (6) CAU-M 16, (7) CAU-M 19, (8) CAU-M 20, (9) CAU-M 24, (10) CAU-M 26, (11) CAU-M 27, (12) CAU-M 28, (13) CAU-M 31, (14) CAU-M 36, (15) CAU-M 52, (16) CAU-M 53, (17) CAU-M 60, (18) CAU-M 66.

at 94°C each cycle then comprised of denaturation at 94°C for 30 s, annealing from 54 to 62°C for 30 s with a reduction of 0.5°C in each cycle, extension at 72°C for 45 s and was repeated for 19 cycles. This was followed by a denaturation for 30 s at 94°C, annealing for 30 s at 60°C, extension for 45 s at 72°C and a final extension of 72°C for 10 min at the end of 20 cycles. The PCR products were then mixed with bromophenol blue and loaded onto a 3% agarose gel and resolved for 3 h and visualized using a gel documentation system (Bio-Rad Laboratories Inc., USA).

Estimation of Total Carotenoids and β -Carotene Contents

Seeds samples were ground well to fine powder and extraction steps were undertaken in dark condition. The carotenoid was extracted as described by the Harvestplus protocol (Rodriguez-Amaya and Kimura, 2004). Quantification of the β -carotene was done using high-performance liquid chromatography (HPLC) system and samples were eluted through the Shimadzu HPLC Analytical C18G 120A column (250 \times 4.6 mm) and detected with

TABLE 1 | Details of *crtRB1* allele, total carotenoids, and β -carotene contents in maize landraces from NEHR of India.

S.No	Gene bank No.	Code ¹	Local name	Place of cultivation	Kernel color and type	<i>crtRB1</i> allele (bp)	Total carotenoids ($\mu\text{g/g}$)	β -carotene ($\mu\text{g/g}$)
1	PBG1107005	CAU-M 12	Minaya	Mizoram	Orange yellow	296 + 543	8.34	1.88
2	PBG1106006	CAU-M 13	Maima	Tripura	Yellow	296 + 543	5.57	1.38
3	PBG1107007	CAU-M 16	Kabullamah	Tamenglong–Manipur	Light red with yellow cap	296 + 543	6.88	4.26
4	PBG1107008	CAU-M 17	Mogadam	Tripura	Light red with yellow cap	296 + 543	2.18	1.36
5	PBG1107010	CAU-M 19	MogadamPaito	Tripura	Brown black	296 + 543	7.16	1.58
6	PBG1208027	CAU-M 60	Vekla	Manipur–Ukhrul	Orange yellow	296 + 543	32.53	3.80
7	PBG1108028	CAU-M 66	Chujak	Manipur–Yairipok	Orange yellow	296 + 543	36.37	4.40*
8	PBG1108018	CAU-M 28	Makkai	Tripura	Violet brown	296 + 543	7.04	2.16
9	PBG1109024	CAU-M 53	Chujak (kabokpokpi)	Manipur–Serou	Orange yellow	296 + 543	16.21	0.74
10	PBG 1404028	CAU M 138	Makei	Sikkim collection	White	296 + 543	5.55	0.20
11	PBG1107009	CAU-M 18	Khamathei	Ukhrul–Manipur	Pale yellow	296	14.39	0.64
12	PBG1107012	CAU-M 20	MakkaiPepe	Arunachal Pradesh	Orange yellow	296	21.4	0.58
13	PBG1106001	CAU-M 1	Meraku	Meghalaya	White	296	3.56	0.36
14	PBG1108016	CAU-M 26	Sohru(Seem)	Meghalaya	Orange yellow	296	17.64	0.84
15	PBG1107013	CAU-M 21	Ambo	Tripura	Orange yellow	296	3.74	0.71
16	PBG1110026	CAU-M 55	Thangkutta	Manipur–Toupokpi, Chandel	Orange yellow	296	20.00	0.94
17	PBG1108020	CAU-M 31	Kolbu	Manipur–Curchandpur	Orange yellow	296	8.37	0.82
18	PBG1108022	CAU-M 39	Chujak	Ukhrul–Manipur	Violet brown	296	6.24	0.38
19	PBG1108015	CAU-M 25	Khamathei	Ukhrul–Manipur	Orange yellow	296	16.58	0.67
20	PBG1106002	CAU-M 2	Chujak	Andro–Manipur	Yellow	296	5.32	0.83
21	PBG1106003	CAU-M 3	Chujak	Khurai–Manipur	Dark red with yellow cap	296	8.12	1.57
22	PBG11060023	CAU-M 52	Block chujak	Serou–Manipur	Yellow orange	296	25.39	0.43
23	PBG1108022	CAU M 36	Kolbu	Churchandpur–Manipur	White/cream	296	21.76	0.86
24	PBG1107014	CAU M 24	Muralimakai	Sikkim	Orange yellow	296	17.97	3.5
25	PBG 1109029	CAU-M 65	Mogadham	Khowai–Tripura	white	296	4.26	1.48
26	PBG1108017	CAU-M 27	Ambo	Ukhrul–Manipur	Dark red	296	1.44	0.33

SEd value: 0.02, CD value ($p = 0.05$): 0.03, CD value ($p = 0.01$): 0.04. *Significantly superior when compared to the other landraces at both 5 and 1%.

a photodiode array detector set at 450 nm. The mobile phase comprising of Acetonitrile: Methanol: Ethyl acetate (80:10:10) at high pressure through the column, and the flow rate was 1 ml min^{-1} . Standards for β -carotene from M/s. Sigma Aldrich India was reconstituted in acetone to five different concentrations (0.1; 1; 10; 50; $100 \mu\text{g/g}$; Kurilich and Juvik, 1999) and was used to construct the standard curve for β -carotene. The β -carotene content in each landrace was measured using standard regression with external standards. The amount of β -carotene was calculated by comparing the chromatogram to that of the standard. The β -carotene content of the 26 landraces were analyzed using Analysis of Variance (ANOVA). The SEd and CD values were also calculated at both 5 and 1% to select for the superior landrace that has significantly higher content of β -carotene.

RESULTS AND DISCUSSION

Maize landraces are one of the major sources for breeding programs due to their unique characteristics and ability to adapt

to various stress environments. Mexican landraces “Tuxpeño” exhibits early maturity, drought-tolerance, and resistance to tropical foliar diseases, and better stalk strength (Gutiérrez-Rodríguez et al., 1998). “Bolita” exhibits drought tolerance and better tortilla-making properties; “Olotillo” exhibits strong performance on poor or unfertilized soils (Benz, 1987). In India, genotypes well-adapted to the hill region and hybrids highly-tolerant to leaf blight were developed using landraces originated from Jammu & Kashmir and Uttarakhand (Prasanna, 2010). NEHR is the center of maize diversity in India. Maize landraces from NEHR possess many valuable agronomic traits. Despite its agronomic benefits, it has remained as an underutilized. In the past decades, only limited attempts have been done to study the features of NEHR maize landraces. Efforts on phenotypic and molecular characterization of a set of 48 maize landrace accessions being made (Sharma et al., 2010). But, studies attempted to characterize the *crtRB1* gene polymorphism and the β -carotene content in the landraces of India are comparatively meager. In particular, no studies have been reported from NEHR maize landraces.

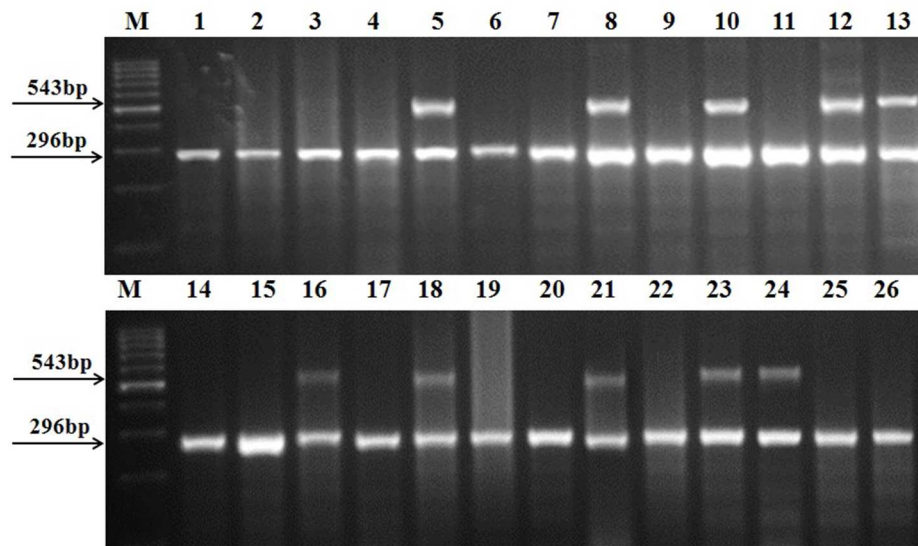


FIGURE 2 | Screening maize landraces from NEHR of India using *crtRB1* gene specific marker. (M) Marker (100 bp), (1) CAU-M 18, (2) CAU-M 20, (3), CAU-M 1, (4) CAU-M 26, (5) CAU-M 12, (6) CAU-M 21, (7) CAU-M 55, (8) CAU-M 13, (9) CAU-M 31, (10) CAU-M 16, (11) CAU-M 39, (12) CAU-M 17, (13) CAU-M 19, (14) CAU-M 25, (15) CAU-M 2, (16) CAU-M 60, (17) CAU-M 3, (18) CAU-M 66, (19) CAU-M 52, (20) CAU M 36, (21) CAU-M 28, (22) CAU M 24, (23) CAU-M 53, (24) CAU M 138, (25) CAU-M 65, (26) CAU-M 27.

MAS was a successful approach that can be used for the rapid selection of the target gene indirectly using molecular markers closely linked to a target gene (Ragot and Lee, 2007; Prasanna et al., 2010). *crtRB1* had a significant effect on enhancing β -carotene content and it exists in three allelic forms Allele 1 (favorable effect), Allele 2, and Allele 3 (unfavorable effect) because of the *crtRB-3'TE* polymorphism that occurs within the gene (Yan et al., 2010). Co-dominant marker developed from the 3'TE region of the *crtRB1* gene facilitated the chance for accelerating the β -carotene improvement programs by MAS (Yan et al., 2010). Recently, many researchers used this allele-based marker to detect the *crtRB1* gene polymorphism in maize germplasms (Muthusamy et al., 2014; Zunjare et al., 2018; Chandran et al., 2019). In the present study, 26 maize landraces from NEHR were characterized for *crtRB1* gene polymorphism using a gene-specific marker (*crtRB1* 3'TE). It showed that none of the maize landraces were homozygous for the favorable allele (543 bp amplicon only), however, the landraces are either heterozygous [both favorable and unfavorable alleles (296 + 543 bp amplicons)] or homozygous [unfavorable allele (296 bp amplicon only)]. Ten maize landraces were found to contain the *crtRB1* gene in the heterozygous form while the remaining 16 landraces were in homozygous form with the unfavorable allele (Figure 2). These results are consistent with the reports of Vignesh et al. (2012), Babu et al. (2013), Selvi et al. (2014), who described that the most favorable *crtRB1* allele is rare in frequency.

Further, we estimated the total carotenoid and β -carotene content in 26 maize landraces. The total carotenoid content in the 26 maize landraces ranged from 1.44 to 36.37 ($\mu\text{g/g}$) showing the diversity among the landraces. CAU-M66 was found to have

a high total carotenoid content of 36.37% followed by CAU-M60 at 32.53%. On the other hand, the β -carotene content varied from 1.36 to 4.40 ($\mu\text{g/g}$) in the heterozygous allele type landraces and 0.33 to 0.94 ($\mu\text{g/g}$) in the homozygous unfavorable allele type landraces. CAU-M66 (4.40 $\mu\text{g/g}$) and CAU-M16 (4.26 $\mu\text{g/g}$) exhibited the highest β -carotene content among the 26 landraces (Table 1). Similarly, Vignesh et al. (2012) revealed that the β -carotene content ranged from 0.02 to 16.50 $\mu\text{g/g}$ in 105 maize inbreds. Selvi et al. (2014) reported that the β -carotene content of the maize inbreds varied from 0.23 to 7.92 ($\mu\text{g/g}$). Muthusamy et al. (2015) also reported that kernel β -carotene ranged from 0.41 to 11.51 $\mu\text{g/g}$ in 14 yellow maize inbreds. Besides, our results also confirmed that the existence of the *crtRB1* favorable allele has a positive correlation with higher β -carotene content. However, Babu et al. (2013) reported that the favorable *crtRB1* allele was found to be more efficient in accumulating higher β -carotene when present in homozygous condition than under heterozygous condition. Thus, in the near future, two maize landraces CAU-M66 and CAU-M16 possess the highest amount of β -carotene content can be selfed for obtaining the homozygous favorable allele and also used as a starting material for the development of new maize varieties rich in β -carotene.

CONCLUSION

This is the first study report the *crtRB1* gene polymorphism and β -carotene content variations in maize landraces originated from NEHR of India. Our study found that two maize landraces CAU-M66 and CAU-M16 have the highest amount of β -carotene content and had the favorable allele in the heterozygous condition among the 26 maize landraces studied. These two

maize landraces are a potential donors that can be exploited in β -carotene biofortification breeding.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

SN, GK, and LM designed the methods and experiments. SM and RM provided suggestions on experiments and monitored the

work. TS, TD, NC, and VS conducted biochemical and genotype analyses. SN, NC, and SC analyzed the data. NC, KA, and SN contributed to the manuscript preparation.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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