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Detecting the Presence of S_H3 Resistance Genes in *Coffea* spp. Cultivars using Genetic Markers

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Abstract: Analyzing the similarities and disparities between *Coffea arabica* and *Coffea eugenioides* offers valuable insights into the evolutionary paths of various traits, including disease resistance. Among the many challenges encountered by coffee growers, coffee leaf rust (CLR) stands out as a significant threat caused by the fungus *Hemileia vastatrix*. This research delved into the genetic foundations of CLR resistance by focusing on identifying S_H3 resistance genes within *C. arabica* ET-39 and *C. eugenioides* Bu-A cultivars, particularly within the Chromosome 3 region. Nucleotide sequences of interest were sourced from an online database, GenBank, and underwent in silico analysis using advanced bioinformatics tools from the R software. Due to the absence of a precise S_H3 gene sequence, the study utilized five associated molecular markers that are commonly used to detect the presence of S_H3 genes: A-48-21O-f, Sat160, Sat180, SP-M16-SH3, and BA-124-12K-f. Three primer pairs—BA-48-21O-f3, Sat160, and Sat281—were detected successfully in the *C. arabica* ET-39 cultivar. In the case of *C. eugenioides* Bu-A cultivar, none of the primers attached within its Chromosome 3 region. These results suggest a higher likelihood of S_H3 resistance genes in *C. arabica* compared to *C. eugenioides*. The relationship between the number of primers attaching to the Chromosome 3 region and the probability of a species harboring S_H3 resistance genes is directly proportional. Consequently, the detection of three primer pairs in the *C. arabica* cultivar, contrasting with the absence in the *C. eugenioides* cultivar, implies a greater potential for CLR resistance in *C. arabica*.

Key Words: *Coffea*; Coffee Leaf Rust; S_H3 Resistance Genes; Chromosome 3; R software

1. INTRODUCTION

Coffee holds significant importance as a valuable commercial crop globally. *Coffea* belongs to the Rubiaceae family, comprising evergreen plants from trees to shrubs. It has funnel-like flowers and has a fleshy cherry fruit that has two seeds—which are the coffee beans themselves. *Coffea* includes 124 varieties (Davis et al., 2011), each with a chromosome count of 2n = 22, except for *C. arabica*, which has 2n = 4x = 44. The innate allotetraploid characteristic of *C. arabica* is made possible by the natural hybridization process between *C. eugenioides* and *C. canephora* (Ingelbrecht et al., 2023). Among the commercially grown species, *C. arabica* is unique in being self-fertile (Lashermes et al., 1999). *C. arabica*, commonly known as Arabica coffee, is cultivated in various countries, including the Philippines—and is preferred over other species of *Coffea* because of its superior quality.

Currently, the primary production of *C. eugenioides* is on Finca Inmaculada farm in Western

Columbia, and it is indigenous to East Africa, the Democratic Republic of Congo, Rwanda, Uganda, Kenya, and Western Tanzania. *C. arabica* and *C. eugenioides* share a close genetic relationship as they belong to the same genus, and are both members of the *C. arabica* complex. Within the *Coffea* genus, despite being separate species, they are closely related. Moreover, *C. eugenioides* is thought to be the maternal progenitor of *C. arabica*, along with *C. canephora* (Balunek et al., 2020). Their genetic relatedness makes them valuable for comparative genomic research to explore how traits like disease resistance evolved. Despite their different species and production disparities, *C. arabica* and *C. eugenioides* share genetic heritage and are potential candidates for coffee breeding programs. *C. eugenioides* beans have not been commercially in demand until recently when the said species shone in the 2021 World Baristas Championship (Walbank, 2022). As a result, the demand for this coffee species increased—calling for the immediate cultivation of the said species.

Worldwide, *C. arabica* is one of the main species for coffee production (Murphy, 1997). However, this species is still susceptible to diseases and infections. According to the study of Ramalho-Bean et al. (2024), Arabica coffee is frequently vulnerable to pest and disease infestations—and enhancing resistance stands as a primary objective within plant-breeding initiatives and research. One devastating coffee disease is coffee leaf rust (CLR), caused by the fungus *Hemileia vastatrix*, which forms orange-yellow pustules on leaves, leading to defoliation and reduced coffee yield (Talhinhas et al., 2017).After the first reported incident of *H. vastatrix* infection in coffee—*C. arabica* crops in Sri Lanka were destroyed, which was devastating as they were known to be the prime coffee producer and exporter (Browning, 1979). Therefore, the need to create CLR-resistant coffee species via cross-breeding is encouraged to prevent such events from happening once again, especially in major coffee-exporting countries.

CLR can be treated with fungicide, but some *Coffea* species possess CLR resistance genes. Within the *Coffea* genus, nine S_H genes (S_H1-S_H9) were discovered, with the S_H3 gene, derived from *Coffea liberica*, exhibiting potent resistance among others (Lashermes et al., 2010). Since the identification of the natural resistance associated with the wildtype *C. liberica*, it has been used to hybridize *C. arabica* in the possibility of acquiring the S_H3 resistance genes.

Cultivars within a species show significant genetic diversity due to efforts in breeding and the selection for traits such as yield, quality, and disease resistance. Examining cultivars provides researchers with a more comprehensive understanding of the specific genetic variations associated with SH3 resistance. Additionally, since growers actually plant and cultivate coffee cultivars, identifying which ones possess resistant genes directly impacts agricultural practices. Thus, informing breeding programs aimed at developing new cultivars with improved resistance to S_H3, ultimately aiding farmers in reducing crop losses caused by CLR. Focusing on cultivars rather than species offers a more precise and practical approach to comprehending and addressing S_H3 resistance in coffee, directly benefiting growers and the coffee industry.

In the study of Mahé et al. (2008), ten genetic markers for the S_H3 gene were identified—BA-42-21B-r, BA-48-21O-f, BA-124-12K-f, Sat160, Sat244, Sat281, SP-M5-SH3, SP-M8-SH3, SP-M16-SH3, and SP-M18-SH3. The determination of these markers permits an easier and cost-effective detection of the S_H3 gene in coffee plants, which in turn becomes useful for breeding programs aiming for the introgression of the resistance genes. Moreover, the study of Silva et al. (2023) showed that SP-M16-SH3 and BA-124-12K-f molecular markers exhibited higher efficacy in detecting the presence of S_H3 within *Coffea* species. According to Santos et al. (2023), S_H3 genes have been mapped in the Chromosome 3 (Chr 3) of the *Coffea* genome. With the existence of *C. arabica* and *C. eugenioides* cultivars, this study sought to identify the presence of the S_H3 gene within the cultivar Chr 3 genomes from GenBank through the R software using BA-48-21O-f, Sat160, Sat180, SP-M16-SH3, and BA-124-12K-f as genetic markers.

The findings of this study are significant for the understanding of the genetic basis of diseases that affect the two commonly grown coffee species particularly in analyzing CLR resistance linked to the S_H3 gene. The prevalence of S_H3 genes in *C. arabica* and *C. eugenioides* cultivars can be compared to gain a deeper understanding of the genetic resistance that underlies the target *Coffea* species and contributes to the validation of the associated markers related to the S_H3 resistance gene. In addition, those who wish to develop disease-resistant coffee cultivars may find great benefit from the study's findings.

By utilizing the boundless capacity of R software's bioinformatics tools in analyzing biological data, this study sought to identify the presence of S_H3 resistance genes in *C. arabica* and *C. eugenioides* cultivars lying in Chr 3 using genetic markers. Specifically, it aimed to determine the presence of S_H3 resistance gene in two *Coffea* cultivars and verify if the molecular markers used for S_H3 detection would anneal to the sequences in the Chr 3 genome. Lastly, this study

aimed to identify and compare the prevalence and genetic variations of the S_H3 resistance gene through markers within *C. arabica* and *C. eugenioides* cultivars.

2. METHODOLOGY

In light of assessing the underlying resistance to CLR in *C. arabica* and *C. eugenioides* cultivars, this methodology aimed to leverage genetic markers derived from *C. liberica* to detect the presence of S_H3 resistance genes in *C. arabica* and *C. eugenioides* cultivars. By employing bioinformatics tools and molecular biology techniques, this study sought to discuss the genetic diversity and potential resistance within *C. arabica* and *C. eugenioides* populations. To identify the presence of S_H3 resistance genes in *C. arabica* cultivars and *C. eugenioides* cultivars, the researchers followed a series of steps primarily utilizing R software—a user-friendly and efficient programming language well-suited for data analysis (see Figure 1). The libraries used in R software for this study are adegenet, ape, ggtree, ggplot2, stats, ips, msa, Biostrings, seqinr, and primerTree.

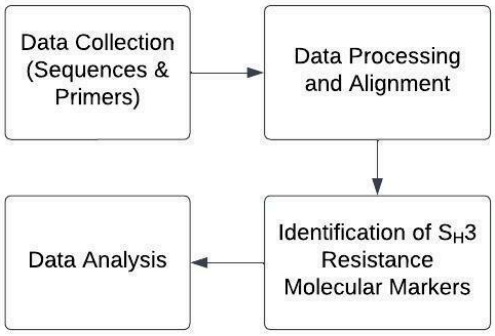

Figure 1. Research Workflow

2.1 Data Collection

Upon searching the GenBank of the National Center for Biotechnology Information (NCBI), the following keywords were used to search for sequences: "Chromosome 3," "Genome," "*Coffea*," and "Cultivar." Nucleotide sequences of interest were obtained using the rentrez::entrez_fetch function. Specifically, sequences for a selected *C. arabica* cultivar ET-39 and *C. eugenioides* cultivar Bu-A, both from the Chr 3 genome, were retrieved in FASTA format (see Table 1). This step ensured access to the genetic information necessary for analysis.

Table 1. *C. arabica* and *C. eugenioides* cultivars used

Coffea spp. Cultivar	Accession ID	Country of Origin	Year Released
C. arabica cultivar ET-39	CM071895.1	France	2024
C. eugenioides cultivar Bu-A	CM071882.1	France	2024

2.2 Data Processing and Alignment

Upon retrieval, the acquired sequences underwent meticulous processing to eliminate extraneous information and standardize the data for downstream analysis. This involved reading the FASTA files and utilizing regular expressions to clean the sequences, removing any extraneous information such as headers and line breaks. The resulting clean sequences were stored as character vectors, facilitating efficient manipulation and analysis in subsequent steps.

2.3 Identification of S_H3 Resistance Gene Molecular Markers

Since there were no available sequences for S_H3, five (5) molecular markers, specifically BA-48-21O-f, Sat160, Sat180, SP-M16-SH3, and BA-124-12K-f, were utilized (see Table 2). Once *C. arabica* cultivar ET-39 and *C. eugenioides* cultivar Bu-A sequences were processed and aligned with the molecular markers specified—the identification of S_H3 resistance genes was performed by looking for motifs based on the molecular markers in the cultivar sequences. Utilizing the matchPattern function, matches for both forward and reverse primers were identified, providing insights into the distribution of S_H3 resistance genes across the Chr 3 region of selected *Coffea* spp. cultivars. The locations of these primers on the sequences were visually depicted through plots generated using the ggplot2 package for analysis.

Table 2. *Markers for S_H3 detection derived from Mahé et al. (2008)*

Molecular Marker	Sequence
BA-48-21O-f	Forward: ACAGTGAATTCCCCAAGCAC Reverse: ACTTGGCAGGCGTAATTGAA
Sat160	Forward: TGCTTAGGCACTTGATATAGGA Reverse: CACGTGCAAGTCACATACTTTA
Sat281	Forward: TCTTCGTCTTTGCTATTGGT Reverse: TATTAACGTCCATCCACACA
SP-M16-SH3	Forward: TTAACTGGAAACTTGGCTTG Reverse: CAAGCCAAGTTTCCAGTTAA
BA-124-12K-f	Forward: TGATTTCGCTTGTTGTCGAG Reverse: TAACGTGCCATCAATCTGCA

2.4 Data Analysis

The results of primer detection were analyzed to determine the genetic landscape of CLR resistance in *C. arabica* cultivar ET-39 and *C. eugenioides* cultivar Bu-A. Comparative analysis was conducted to assess the prevalence of S_H3 resistance genes across different cultivars and their alignment through the plots generated in the R software. Interpretation of the findings offers insights into genetic variation and potential resistance within *C. arabica* and *C. eugenioides* populations.

3. RESULTS AND DISCUSSION

In the pursuit of understanding genetic factors underlying resistance to *H. vastatrix*, S_H3 in *C. arabica* and *C. eugenioides* cultivars were identified within their Chr 3 genome. According to Santos et al. (2023), the S_H3 region has been mapped in the Chr 3 of CLR-resistant coffee species, including *C.liberica*, *C. canephora*, and *C.arabica* var Caturra. With the discovery of the inherent resistance mechanism of S_H3 in *C.liberica*, the species is subjected to selective breeding with other susceptible coffee species to introgress or pass S_H3 to their offspring (see Figure 2). As a result, various cultivars of commercially demanded coffee species, such as *C. arabica* and *C. eugenioides* were produced. Furthermore, this region is highly conserved, suggesting that CLR-resistant coffee species should also possess the genetic markers being identified by primer pairs within the Chr 3. Through the utilization of R software, this study was able to identify the S_H3 region using established primer pairs in silico—indicating the possibility of the cultivars expressing resistance phenotypes against CLR.

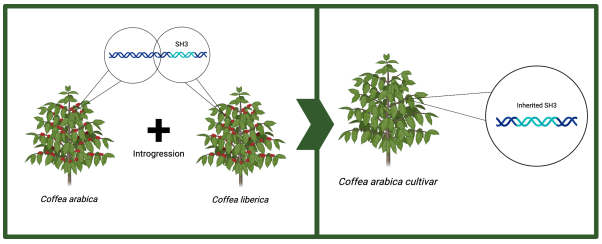

Figure 2. Process of Introgression (Figure made from Biorender.com)

3.1 Detection of S_H3 Region in C. arabica cultivar ET-39

Among the five primer pairs used in this paper, the BA-48-21O-f, Sat160, and Sat281 successfully aligned within the Chr 3 of *C. arabica* cultivar ET-39. The matches recorded using these primer pairs can be used as indicators to confirm the presence of SH3 region—most importantly, the possibility of the cultivar being resistant against CLR. The BA-48-21O-f primer pair has forward and reverse primers with 20bp length. As shown in Figure 3, they were able to bind within the Chr 3 of *C. arabica* cultivar ET-39. Furthermore, Table 3 summarizes the positions of the BA-48-21O-f primer pair within the sequence. Similarly, in the study conducted by Merlin (2023), the BA-48-21O-f primer pair also aligned at around 3 Mbp of the Chr 3 genome of *C. canephora.*

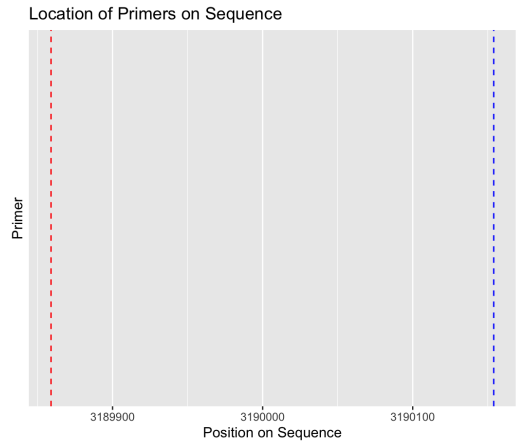

Figure 3. Location of BA-48-21O-f Primer Pair on *C. arabica* cultivar ET-39 Chr 3 sequence (Note: Red line = forward primer; Blue line = reverse primer)

Moreover, the Sat160 primer pair has a base length of 22 bp. Based on Table 3, the position of Sat160 is located around 8 Mbp. In the study of Merlin (2023), the same primer pair was also observed to have binding sites around 8 Mbp of the Chr 3 of *C. canephora*. The binding sites of the primers within the Chr 3 of *C. arabica* cultivar ET-39 can be visualized in Figure 4.

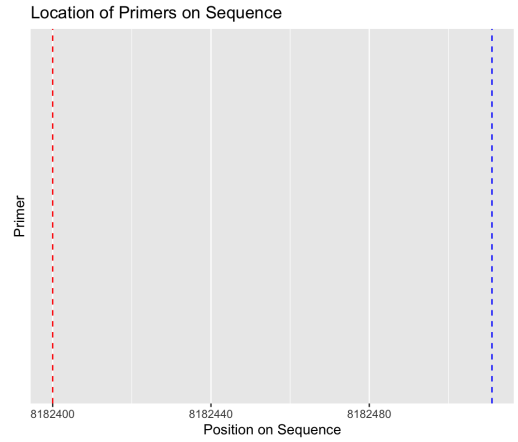

Figure 4. Location of Sat160 Primer Pair on *C. arabica* cultivar ET-39 chr 3 sequence (Note: Red line = forward primer; Blue line = reverse primer)

On the other hand, the Sat281 primer pair has a length of 20bp. As shown in Table 3, the binding sites of both forward and reverse primers are located at around 11 Mbp. These are further visualized in Figure 5. Similarly, the findings of Merlin (2023) present that this primer pair also annealed at around 11 Mbp of the Chr 3 of *C. canephora*.

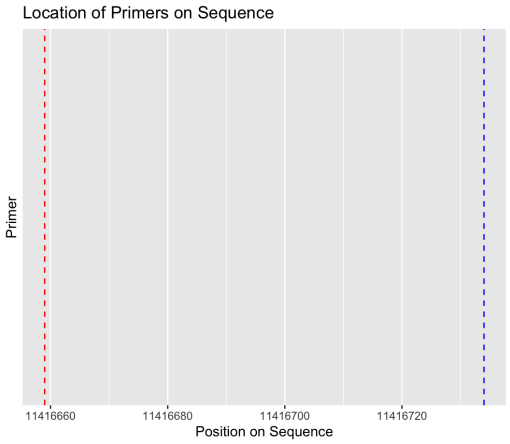

Figure 5. Location of Sat281 Primer Pair on *C. arabica cultivar* ET-39 Chr 3 sequence (Note: Red line = forward primer; Blue line = reverse primer)

Therefore, the comparable binding sites of these primer pairs in both *C. canephora*, as shown in the study of Merlin (2023), and *C. arabica* cultivars support the claim that the S_H3 region is conserved among CLR-resistant coffee species. Moreover, the variances in terms of the positions of the binding sites of the primer within Chr 3 support the study of Cui et al. (2020), which also showed that binding sites of different markers are disseminated throughout Chr 3 of the coffee genome. Therefore, this implies that the S_H3 is not a single gene but a region composed of multiple genes spread throughout the Chr 3 (Santos et al., 2023). Furthermore, Merlin (2023) also highlighted that the S_H3 falls between 3 Mbp to 16 Mbp of the Chr 3 of *Coffea* species, which aligned with the results of this study.

Other primer pairs used were SP-M16-SH3 and BA-124-12K-f. However, using the pipeline in this study, these primers did not return any matches within the Chr 3 of the *C. arabica* cultivar ET-39. As a result, the findings of this paper do not support the claims of the study conducted by Silva et al. (2023)—suggesting the SP-M16-SH3 and BA-124-12K-f are the most efficient markers to be used, especially SP-M16-SH3 as they are codominant. The study by Merlin (2023) also had the same results—the SP-M16-SH3 was not able to bind to the Chr 3 of *C.canephora* in silico.

Table 3. Position of Primers in the *C. arabica* cultivar ET-39

Primers		Start Position (bp)	End Position (bp)
BA-48-21O-f	Forward	3,189,859	3,189,878
	Reverse	3,190,154	3,190,173
Sat160	Forward	8,182,400	8,152,421
	Reverse	8,182,511	8,182,532
Sat281	Forward	11,416,659	11,416,678
	Reverse	11,416,734	1,416,753

3.2 Detection of S_H3 Region in C. eugenioides cultivar Bu-A

In the study conducted by Angelo et al. (2023), four gene variants of S_H3 were identified within the Chr 3 of *C. eugenioides* and a highly conserved S_H3 gene variant in Chromosome 6. Thus, indicating the ability of *C. eugenioides* to persist against CLR. In contrast, the five chosen primers in this study were not able to bind to any site within the Chr 3 of the *C. eugenioides* cultivar Bu-A that was obtained from France. This indicates that the S_H3 region, specifically in Chr 3, is not present for this cultivar. Thus, it is also possible that this cultivar does not possess the resistance phenotype against CLR unless other chromosomes are screened for possible S_H3 variants that confer resistance.

In a study by Yuyama et al. (2015) analyzing the transcriptome of *C. eugenioides* as an ancestor of *C. arabica, C. eugenioides* is found in highland areas and near forest edges across Central-East Africa, but its commercial cultivation is limited due to its low fruit yield. In breeding programs, it has been targeted for its potential to reduce caffeine levels and enhance cup quality, owing to its production of small fruits with lower caffeine content compared to both *C. arabica* and *C. canephora*. Despite being recognized as the maternal progenitor of *C. arabica*, the absence of all primers in *C. eugenioides* in the study was contradictory. However, one explanation could lie in the asymmetrical contribution of the two subgenomes present in the allotetraploid genome of *C. arabica* (subgenome *C. canephora*—CaCc and subgenome *C. eugenioides*—CaCe) (Vidal et al. 2010; Cotta et al. 2014). Studies suggest that these subgenomes exhibit differential expression patterns, with CaCc more involved in regulatory protein expression and CaCe associated with basal processes. This implies that the observed markers in *C. arabica* may not solely originate from its ancient maternal progenitor, necessitating consideration of both progenitors. The unequal contributions of these progenitors could potentially explain the findings of the study.

Another aspect to consider is the exposure of *C. arabica* to different *Coffea* species or varieties. As previously mentioned, *C. arabica* is significantly more cultivated compared to *C. eugenioides*. As indicated by Clayton (2021), while *C. eugenioides* plants are not inherently more challenging to cultivate, they do yield less than *C. arabica*. Moreover, *C. eugenioides* is still regarded as rare or experimental in the realm of coffee farming, until recently. Consequently, *C. arabica* is more likely to interact with other *Coffea* species. The study of Alkimim et al. (2017) reported that the S_H3 gene of the wildtype *C. liberica* has been introgressed into *C. arabica*, leading to the creation of *C. arabica* cultivars expressing resistance against CLR. Furthermore, the widespread presence of *C. arabica* across varied environments and the decrease in diploid competitors might have facilitated the divergence of initially similar populations, ultimately contributing to the evolution of *C. arabica* as a distinct species (Merot-L'anthoene et al., 2019). This heightened exposure and genetic variability could have increased the likelihood of *C. arabica* possessing genes like S_H3 for CLR resistance.

The findings of this study primarily indicated the presence of markers linked to the S_H3 resistance gene in *C. arabica* cultivar ET-39 and its absence in *C. eugenioides* cultivar Bu-A. Thus, the results of this study serve as a foundation for the genetic basis of CLR resistance of *Coffea* cultivars. Further studies regarding external factors like climate and environment should be conducted to supplement the existing knowledge regarding CLR resistance of *Coffea* cultivars.

4. CONCLUSIONS

The advancement in bioinformatics tools provides researchers with countless opportunities to analyze large amounts of biological data. Through the use of automated and modern bioinformatics tools in R software, results revealed significant differences in the detection of molecular markers targeting S_H3 resistance genes between *C. arabica* and *C. eugenioides* cultivars. Specifically, the binding sites for

primer pairs BA-48-21O-f3, Sat160, and Sat281 were successfully detected in *C. arabica* cultivar ET-39 but were absent in *C. eugenioides* cultivar Bu-A. This discrepancy suggests potential genetic variations between the two *Coffea* species regarding the presence of the S_H3 resistance genes. Furthermore, the inability of primer pairs SP-M16-SH3 and BA-124-12K-f to attach to any of the *Coffea* species indicates either the absence of these specific genetic motifs in the studied cultivars or technical limitations in primer design and detection.

The relationship between the number of primers attaching to the Chr 3 region and the likelihood of a species harboring S_H3 resistance genes is directly proportional. Therefore, the detection of three (3) primer pairs, BA-48-21O-f3, Sat160, and Sat281, in *C. arabica* cultivar compared to none in *C. eugenioides* cultivar suggests a higher probability of the presence of S_H3 resistance genes in *C. arabica* cultivar—making it more CLR resistant. The results of this study proved the success of multiple cross-breeding of *C. arabica* with other CLR-resistant coffee species because of the S_H3 presence in the ET-39 cultivar. Thus, allowing the proliferation of more *C. arabica* cultivars. On the other hand, this study also urges further research and studies regarding *C. eugenioides* in general as they are recently considered as emerging species for coffee bean production. In addition, external factors such as climate and environment should also be studied to support the existing information about the CLR resistance in *Coffea* cultivars.

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