Endophytic bacterial community diversity in two citrus cultivars with different citrus canker disease resistance

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Abstract
The selective infection of *Xanthomonas citri* pv. *citri* (*Xcc*) to citrus cultivars is universally known, but the relationship between endophytic bacteria and the resistance of host variety to canker disease remains unclear. In this study, endophytic bacterial populations of two citrus cultivars—the resistant satsuma mandarin and the susceptible Newhall navel orange—were analyzed through high-throughput sequencing. The results showed that endophytic bacterial community of satsuma mandarin was more abundant than that of Newhall navel orange. In addition, bacterial abundance was the highest in the spring samples, followed by that in summer and winter samples, in both the varieties. In all samples, the predominant phyla were Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes; the major genera were *Bacillus* and *Stenotrophomonas*, and the main species was *Bacillus subtilis*. According to the analysis of the predominant bacteria in the two citrus cultivars, *B. subtilis* with potential antagonistic characteristics against *Xcc* existed universally in all samples. However, the susceptible Newhall navel oranges were abundant in *Bacillus subtilis* and had a relatively large number of canker-causing cooperative bacteria such as *Stenotrophomonas*. The results suggested that endophytic bacterial community of the two citrus cultivars had some differences based on the season or plant tissue, and these differences were mainly in the quantity of bacteria, affecting citrus canker disease occurrence. In conclusion, the differences in endophytic bacteria on citrus cultivars might be related to host resistance or susceptibility to citrus canker disease.

Keywords Citrus canker · Resistance · Susceptibility · Endophytic bacterial diversity

Introduction
Plant endophytic bacteria represent a class of microorganisms living within plant tissues and organs without causing any disease or symptoms. Both endophytic bacteria and host plants adapt to and interact with each other to form complex symbiotic relationships. The plant genetic backgrounds and genes have a significant effect on the endophytic bacterial species, their quantity, and their activity (Hamilton et al. 2010; Gundel et al. 2012). Plant endophytic bacteria also have a degree of feedback on host growth (Nair and Padmavathy 2014). In recent years, endophytic bacteria have gained considerable attention because they have been found to enhance host plant tolerance to stressful environments, promote plant growth, and improve plant protection (Bulgarelli et al. 2013; Ying et al. 2006). However, the aforementioned knowledge, particularly regarding plant pathogen growth inhibition and disease resistance, is mostly specific to culturable endophytes alone (Yuan et al. 2005; Liu et al. 2011; Zhao et al. 2016; Mnasri et al. 2017; Akbaba and Ozaktan 2018; Yousefi et al. 2018). The most bacteria in plants that cannot be cultured on artificial media have a vital influence on host growth including that in disease resistance (Sun and Song 2006). Therefore, the diversity of endophytic bacterial community warrants exploration so as to deeply understand endophyte–host interactions.

Citrus canker disease caused by *Xanthomonas citri* pv. *citri* (*Xcc*) is an important quarantine disease in China. Globally, it has caused large economic losses and shown an increasing trend of spread in the domestic and foreign navel orange cultivation areas (Furman et al. 2013). It can...
demolish an entire citrus industrial operation because of Xcc prevention and control measures are unavailable (Gottwald 2007); therefore, it is called “citrus canker” (Das 2003). Xcc causes a selective infection in citrus cultivars; in other words, citrus canker does not occur in some cultivars such as Nanfeng tangerine, satsuma mandarin, and kumquat citrus, with the disease being relatively more serious in sweet orange. Some citrus cultivars show canker disease resistance, associated with many factors including citrus epidermal factors tissue (especially stomatal frequency) (Wang et al. 2011; Li et al. 2013), physiological components (Gogo et al. 1979), oxidase content (Wang et al. 2011), phytoalexin content (Boddu et al. 2004), resistance genes (Shiotani et al. 2008), as well as possible contribution of culturable endophytic bacteria from citrus (Liu et al. 2013). However, few studies thus far have reported on the relationship between citrus endophytic bacterial communities and the resistance of their host cultivars to canker disease.

In this study, we collected samples from the following two citrus cultivars: Newhall navel orange (Citrus sinensis Osbeck) and satsuma mandarin (Citrus unshiu Marc.), which are susceptible and resistant to canker disease, respectively. The following hypotheses were made:

(i) Host resistance to canker disease determines the diversity and function of the endophytic bacterial community associated with a citrus cultivar.

(ii) Predominant endophytic bacterial communities from citrus cultivars with differences in their resistance to canker disease have distinctive operational taxonomic units (OTUs).

(iii) Endophytic bacteria can be beneficial to host plants in canker disease resistance.

To test these hypotheses, we examined endophytic bacterial communities in the leaves and fruits of the aforementioned citrus cultivars in spring, summer, and autumn through high-throughput sequencing of the 16S rDNA V3-V4 region.

**Materials and methods**

**Sample collection**

Young fruits and tender leaves that did not show any disease symptoms were randomly collected from trees of the canker disease-resistant satsuma mandarin and the canker-susceptible Newhall navel orange in a citrus orchard in Jiangxi Agricultural University, Jiangxi Province, China. The young fruits from the Newhall navel orange and satsuma mandarin trees were denoted as CSN.F and CU.F, respectively; moreover, the tender leaves collected in spring, summer, and autumn were denoted, respectively, as CSN.SP, CSN.SU, and CSN.AU if they were from the Newhall navel orange trees and CU.SP, CU.SU, and CU.AU if they were from the satsuma mandarin trees. Each sample was created by pooling with six leaves or fruits from six independent plants and stored at 4 °C in sterile plastic bags until further analysis.

**Samples preparation and DNA extraction**

To ensure that the microbial communities were endophytes, the samples were surface sterilized using 70% alcohol for 1 min and 1% sodium hypochlorite solution for 1–5 min before DNA extraction. Samples were then washed four times with sterile water and dried on sterile paper. About 0.1 mL of the final eluate was collected to check for bacterial contamination.

Microbial DNA was extracted from plant tissues by using the methods reported by Jiao et al. (2010) and Wu et al. (2018). In brief, plant tissue was ground using liquid nitrogen and mixed well. To exclude the interference of plant chloroplast in the subsequent bacterial genomic DNA extraction, 3 g of ground tissue powder was suspended in 15 mL of respective enzyme solutions (1.5% macerozyme R-10, 1.5% cellulase R-10, 0.12% N-morpholino ethane sulfonic acid, 0.36% CaCl₂·2H₂O, 12.8% D-mannitol, 0.011% NaH₂PO₄, pH 5.6), followed by incubation at 37 °C with gentle agitation for 3 h. The mixture was subsequently centrifuged at 200 g for 5 min with three repetitions. The supernatants were collected and centrifuged at 16,500 g for 20 min, and the resulting pellet was collected. Finally, DNA was extracted using the EasyPure Bacteria Genomic DNA kit according to the manufacturer’s instructions. Each sample analysis was repeated in triplicate.

**DNA amplification and next-generation sequencing**

Purified DNA was used as the template for polymerase chain reaction (PCR)-based amplification. The V3-V4 variable regions of 16S rDNA gene were amplified by the primer pair 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGTATCTAAT-3′) (Caporaso et al. 2011). PCR was performed in a 25-μL reaction system, comprising 2 μL of the diluted template DNA, 8.5 μL of sterile water, 1 μL of each primer, and 12.5 μL of PCR Mix form TaKaRa (Dalian, China). The PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, annealing at 54 °C for 60 s, and extension at 72 °C for 2 min, and last, final extension at 72 °C for 10 min.

The quality of the amplified PCR products was checked through 2% agarose gel electrophoresis. Samples with a bright main strip between 400 and 500 bp were selected for further experimentation. The PCR products were purified using a Qiagen Gel Extraction Kit (Qiagen, Germany) and
then sequenced by Novogene Bioinformatics Technology (Beijing, China) on a HiSeq 2500 platform (Illumina, San-Diego, CA, USA).

**Sequence data treatment and statistical analyses**

Sequencing reads were assigned to each sample according to the unique barcode of each sample. Pairs of reads from the original DNA fragments were then merged on FLASH V1.2.7 (Caporaso et al. 2010). Then, the generated raw tags were filtered using QIIME (Quantitative Insights Into Microbial Ecology) V1.7.0 (Caporaso et al. 2011) and subjected to a quality control procedure on Uparse v7.0.1001. Clean reads were then clustered to generate OTUs at a 97% similarity level. We selected a representative sequence for each OTU and used the ribosomal database project classifier to assign taxonomic data to each representative sequence (Wang et al. 2007). MUSCLE (version 3.8.31) was used to analyze alpha-diversity (within samples) and beta diversity (among samples) (Edgar 2004).

Heatmap analysis with rarefaction curve creation was performed on R2.15.3. The histograms were created on Microsoft Excel 2010. All sequences have been deposited in the NCBI Sequence Read Archive database (accession number: PRJNA515173).

**Results**

**OTU number analysis**

After quality filtering and chimera sequence removal, a total of 1,197,612 effective sequences of eight groups were obtained. The OTUs of different samples were analyzed on the basis of Venn diagrams. The OTUs were abundant in the fruit and leaves (all three seasons) of satsuma mandarin and Newhall navel orange (Fig. 1; Fig.S1). The results showed that these eight samples shared 74 OTUs—indicating that these OTUs may be the relatively stable components in endophytic bacterial communities associated with the two citrus cultivars. Moreover, relatively more unique OTUs were obtained in CN.SP, CSN.SU, CU.SP, and CU.SU samples, indicating high richness of endophytic bacterial communities in spring and summer leaf samples (Fig.S1).

Compared with the different seasonal samples, CN.SP and CU.SP samples had more unique OTUs (332 and 340, respectively). The number of leaf sample OTUs was the highest in spring, followed by that in summer and autumn. In leaf samples from the same season (except for autumn samples), the total number of OTUs was higher in satsuma mandarin than in Newhall navel orange (Fig. 1A, B). Among the fruit samples, CU.F had the highest number of unique OTUs (Fig. 1C). Among different citrus species samples in the same season, spring leaves had 410 shared OTUs, but summer and autumn leaves had only 160 shared OTUs. The number of unique OTUs in spring and summer samples of satsuma mandarin was more than those of Newhall navel orange, but an opposite observation was noted in the autumn (Fig. 1D–F).

**Endophytic bacterial community composition**

All the obtained sequences were classified from the phylum to genus levels according to the program Mothur (version 1.39.3) using the default setting. Mainly ten phyla were identified from these samples (Fig. 2; Table S1). Proteobacteria, Cyanobacteria, and Firmicutes were the most prominent phyla, accounting for >90% of the reads at the phylum level. Moreover, Actinobacteria accounted for a large proportion of the sequences in CSNS.P, CU.SP, CSN.SU, CU.SU, CSN.AU, and CU.AU. In addition, Bacteroidetes account for 1–4% of the sequences in CU.SP, CSN.SU, CU.SU, and CSN.AU. We also found a similar trend of microbial growth and decline in leaf samples of both citrus cultivars in all the sampled seasonal variation. However, their contents of endophytic bacteria phyla differed. For example, Firmicutes contents of the two citrus leaves were the highest in autumn but the lowest in summer, with the bacterial contents being higher in the leaves of satsuma mandarin than in those of Newhall navel orange.

The bacterial composition of the eight samples at the class, order, and family levels is shown in Fig. S2. At the genus level, “Others” (the sum of the relative abundance of all the genera except the top 10) occupied a large percentage. The most abundant genus in leaves and fruits samples (except CU.F and CSN.SU) was Bacillus. Stenotrophomonas, Halomonas, Shewanella, and Brevundimonas were also abundant compared with other genera in different samples (Fig. 3; Table S2). A heatmap was used to analyze the variety of the abundance of most genera in these samples (Fig. 4): Curtobacterium, Knoellia, and Rhizobradyium were predominant in CSN.SP, Propionibacterium, Gardnerella, and Brevundimonas in CSN.SU; Bifidobacterium, Faecalibacterium, Blautia, and Lactobacillus in CU.SP; Brucella, Rhizobium, and Pseudomonas in CU.SU; and Bacillus in CU.AU. In all fruit samples and CSN.AU, there were no predominant genera. In the citrus canker disease-resistant satsuma mandarin, Bacillus, Halomonas, Shewanella, Pseudomonas, and Stenotrophomonas were highly abundant in leaf samples but were less abundant in fruit samples. Thus, the bacterial communities in not only leaves but also fruits of both the cultivars showed obvious difference in dominant genera, regardless of the season.

The top nine species in all samples included *Bacillus subtilis*, *Pseudomonas geniculata*, *Pseudomonas antarctica*, *Shewanella algae*, *Brevundimonas diminuta*,...
Bradyrhizobium elkanii, Massilia timonae, Rhodococcus erythropolis, and Sphingobium yanoikuyae (Table 1). B. subtilis (15.4158–69.6661%) was the most abundant in all samples except CU.F and CSN.SU. The relative abundance of P. geniculata in the summer and autumn samples was much higher than that in other samples. The relative abundance of P. antarctica was the highest in CU.SU, whereas that of Bre. diminuta, M. timonae, R. erythropolis, and S. yanoikuyae was the highest in CSN.SU. Moreover, the relative abundance of Bra. elkanii was the highest in CSN.SP. S. algae was found only in CSN.SP, CU.SP, CSN.SU, and CU.F. B. subtilis had higher abundance in the leaf samples of satsuma mandarin than in those of Newhall navel orange; they were also more abundant in autumn samples than in other seasons’ samples.

**Diversity analysis of endophytic bacterial community**

In the diversity analysis, the same box was used to present the Weighted Unifrac and Unweighted Unifrac, indicating the upper and lower values, respectively. The number in the
chart was the phase coefficient between the samples; the smaller the different coefficients, the smaller the diversity of species. The coefficient of dissimilarity between the two citrus cultivars was 0.228 (0.283) in spring, 0.253 (0.636) in summer, and 0.226 (0.500) in autumn—indicating that the difference in the endophytic bacterial communities between the two citrus cultivars in summer was the maximum (Fig. 5). The dissimilarity coefficient of two citrus cultivars indicated that the differences in the leaf endophytic bacteria in satsuma mandarin and Newhall navel orange had changed greatly with the seasonal variation. Similarly, the coefficient of 0.355 (0.598) revealed the dissimilarity of endophytic bacterial community between the two citrus fruit samples. In satsuma mandarin, the coefficient of dissimilarity between fruit and leaf samples was 0.552 (0.644), 0.526 (0.625), and 0.628 (0.653) in spring, summer, and autumn, respectively, which indicated that the differences in endophytic bacteria were obvious in different tissues of the same citrus cultivar (Fig. 5).

**Discussion**

Few studies thus far have investigated the relationship between endophytic bacteria and host disease resistance but via the traditional isolation and cultivation methods only (Lacava et al. 2007; Flores et al. 2013). However, the microorganisms identified via the traditional methods account for only 0.1–10.0% of the total environmental organisms, which cannot truly reflect endophytic bacterial community
structure (Gangaiah et al. 2009; Magajna and Schraft 2015). In different citrus trees, the bacterial communities differ based on the cultivar. Liu et al. (2013) reported that the quantity and proportion of culturable endophytic bacteria are higher in canker disease–resistant citrus than that in the susceptible citrus. However, very few studies have reported on the unculturable endophytic bacteria. In this study, the diversity and relationship of bacterial community in citrus trees including the canker disease-resistant satsuma mandarin and the canker disease-susceptible Newhall navel orange were analyzed via high-throughput sequencing.

High-throughput sequencing—a milestone in the development of sequencing technology—provides accurate transcripts and genomes up to the species level; accordingly, it also called deep sequencing or next-generation sequencing (Endrullat et al. 2016; Montoya et al. 2016; Yong et al.

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**Table 1** Abundance of the top nine species in the two citrus cultivars (%)

| Bacterial species                   | Samples         |
|-------------------------------------|-----------------|
|                                     | CSN.SP | CU.SP | CSN.F | CU.F | CSN.SU | CU.SU | CSN.AU | CU.AU |
| *Bacillus subtilis*                 | 15.4158 | 35.933 | 38.857 | 0.9515 | 1.2386 | 23.2776 | 41.0932 | 69.6661 |
| *Pseudomonas geniculata*            | 0.3577  | 0.3479 | 0.9779 | 0.3704 | 15.795 | 7.7765  | 5.1513  | 2.4008  |
| *Pseudomonas antarctica*           | 0.0921  | 0.2489 | 0.2499 | 0.0049 | 0.1764 | 8.144   | 0.5105  | 0.0216  |
| *Shewanella algae*                 | 4.7104  | 4.5507 | 1.1171 | 0.0431 | 0      | 0       | 0       | 0       |
| *Brevundimonas diminuta*           | 0.0774  | 0.0412 | 0.148  | 0.0627 | 6.2312 | 3.6276  | 2.0921  | 0.3743  |
| *Bradyrhizobium elkanii*           | 2.5174  | 0.731  | 0.0039 | 0.4253 | 0.0617 | 0.0627  | 0.0598  | 0.0127  |
| *Massilia timonae*                 | 0.097   | 0.049  | 0.1862 | 0.098  | 3.1523 | 1.4993  | 1.1259  | 0.3626  |
| *Rhodococcus erythropolis*         | 0.1137  | 0.0451 | 0.1254 | 0.0235 | 2.2361 | 1.5071  | 1.9059  | 0.6624  |
| *Sphingobium yanoikuyae*           | 0.0343  | 0.0333 | 0.1107 | 0.05   | 3.1768 | 1.4718  | 0.6262  | 0.1872  |

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[![Fig. 4 Heatmap of the relative abundance of the top 35 genera in all samples. These 35 genera belong to five phyla, as shown by different colors. The color intensity within the index in the right corner of the figure indicates the relative values for each genus. The cluster tree on the left side of the graph indicates the clustering between the same phyla.](image-url)](image-url)
Currently, the 16S rDNA amplicon sequencing has been widely used in microbial community analysis in different environments, involving multiple fields (Sun et al. 2014; Shen et al. 2015; Lu et al. 2016; Zhao et al. 2017). In this study, high-throughput 16S rDNA sequencing was used to study the differences of endophytic bacterial community in the two aforementioned citrus cultivars. However, in preliminary testing, high chloroplast, mitochondria, and bacteria homology in phylogenetic sequencing led to most sequences becoming attributable to host chloroplasts and mitochondria after high-throughput sequencing of 16S rDNA V3-V4 variable region. To reduce the proportion of the host pollution phenomenon, we performed enrichment treatment of endophytic bacteria, where the plant cell walls were removed and more endophytic bacteria were released by using cellulase and macerozyme; next, the plant protoplasts and bacteria were separated by differential centrifugation (Wu et al. 2018). However, the proportion of chloroplast and mitochondria in satsuma mandarin fruit peel remained high; the host pollution phenomenon also still appeared in other samples. Therefore, a more appropriate method for enriching endophytic bacteria, selecting more differential primers, or formulating a better sequencing strategy was explored so as to avoid chloroplast and mitochondria interference during 16S rDNA amplicon–based high-throughput sequencing.

The results revealed 74 OTUs shared in all the samples, indicating the presence of identical endophytic bacteria these samples. The order of leaf endophytic bacterial abundance was spring > summer > autumn. We speculated the main reason for this to be spring being able to provide more nutrition. In summer, the growth of citrus was influenced by high temperature, which may lead to decrease in endophytic bacterial abundance. In orange trees, the physiological activity slows in autumn and endophytic bacterial abundance decreases again (Liang et al. 2005; Rodriguez et al. 2017). Here, although the number of OTUs in satsuma mandarin demonstrated large declines as the season progressed, it was higher than that in Newhall navel orange in the spring and summer samples. However, in autumn samples, the trends of the number of OTUs in the two citrus cultivars were opposite. In different cultivars, endophytic bacterial abundance was nevertheless different. Therefore, whether there is a relationship between the dynamic change of citrus endophytic bacteria and the host resistance to cancer disease remains unclear, warranting further exploration by analyzing the endophytic bacteria from the resistant and susceptible citrus trees.

The differences in endophytic bacterial community of samples were significant. At the phylum level, the citrus leaves mainly had Proteobacteria, Firmicutes, and Actinobacter—corroborating the results of Gagne-Bourgue et al. (2013) and Li et al. (2009). Some species of Firmicutes have an inhibitory effect on Xcc causing the citrus canker disease via various process including antimicrobial peptide production, bacteriolysis, and niche competition (Chen et al. 2008, 2014; Huang et al. 2012; Liu et al. 2015). In the current study, Firmicutes abundance was significantly higher in satsuma mandarin leaves than in Newhall navel orange leaves during citrus growth—in accord with the observations of Liu...
et al. (2013). Firmicutes abundance the lowest in all summer samples, with this number being much lower in satsuma mandarin. Consequently, we speculated that summer would be the period with the highest citrus canker disease risk; in order to resist pathogenic bacterial invasion, the Firmicutes in the competing position and interacting with pathogenic bacteria caused a sharp reduction in their population. Therefore, we speculated that the content difference and variation trends of endophytic bacteria attributable to Firmicutes may reflect the canker disease resistance or sensitivity differences among the citrus cultivars. Moreover, the dynamic change in the endophytic bacterial communities may indicate the course of canker disease resistance in citrus, indicating that the differences in the citrus endophytic bacterial community is related to the host citrus canker disease resistance or susceptibility.

At the genus level, endophytic bacteria from two citrus cultivars detected were mainly distributed in Bacillus and Pseudomonas in this study. Bacillus has strong resistance to other harmful factors and is the main functional genus improving host disease resistance and producing active substances such as subtilisin, polymyxin, nystatin, and gramicidin—which have a strong inhibitory effect on pathogens (Rakotoniriana et al. 2013; Ji et al. 2015; Zhang et al. 2016). B. subtilis, Bacillus amyloliquefaciens, and Pseudomonas protegens have been reported to efficiently control citrus canker disease in citrus plants (Huang et al. 2012; Michavila et al. 2017; Sudyoung et al. 2019). Here, we found that Bacillus abundance in the leaves of the two citrus species fluctuated with season—decreasing in summer and increasing in autumn. Nevertheless, the bacterial abundance in satsuma mandarin was higher than that in Newhall navel orange (Table S2; Fig. S2). In particular, Bacillus was only relatively abundant in the autumn leaves of satsuma mandarin, and the presence of the bacterium was possibly a factor that interfered with the pathogenic bacteria to infect citrus in autumn. Some studies have shown that Pseudomonas has an antagonistic effect on Xcc (Zhang et al. 2007; Murate et al. 2015; Michavila et al. 2017). In the current study, the abundance of Pseudomonas was the highest in summer samples; in particular, it was higher in satsuma mandarin samples than in Newhall navel orange samples. This may be reason that satsuma mandarin is not infected by Xcc in summer. In addition, Curtobacterium sp. have been reported to be abundant in sweet orange roots uninfected by Huanglongbing (HLB) pathogen (Trivedi et al. 2011); in particular, Curtobacterium flaccumfaciens can effectively inhibit citrus variegated chlorosis (caused by Xylella fastidiosa) (Araujo et al. 2002). Here, in spring, Curtobacterium was abundant in the leaves of Newhall navel orange but not in those of satsuma mandarin. Inhibition of Curtobacterium may thus be related to the occurrence of canker disease in Newhall navel orange. There was a synergistic effect reported between Stenotrophomonas abundance and canker disease occurrence (Gao 2016). Moreover, Stenotrophomonas was the most abundant in Newhall navel orange leaves in summer, during which the most serious canker disease occurred. Notably, Acinetobacter has been reported to be widely exist on plant leaf surface and has a potential control effect on canker disease (Gao 2016). In this study, Acinetobacter was found to have higher relative abundance in Newhall navel orange leaves in summer, which may indicate the microbial community adjustment occurring in citrus after Xcc infection.

In conclusion, the current results indicated that the differences in endophytic bacterial communities and their abundance in citrus cultivars may be related to the host canker disease resistance or susceptibility.

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Availability of data and material All data generated or analyzed during this study are included in this published article and its supplementary information files.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent to publication Written informed consent for publication was obtained from all participants.

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