The additive function of \textit{YIGE2} and \textit{YIGE1} in regulating maize ear length

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SUMMARY

Ear length (EL) is a key trait that greatly contributes to yield in maize. Although dozens of EL quantitative trait loci have been mapped, very few causal genes have been cloned, and the molecular mechanisms remain largely unknown. Our previous study showed that \textit{YIGE1} is involved in sugar and auxin pathways to regulate ear inflorescence meristem (IM) development and thus affects EL in maize. Here, we reveal that \textit{YIGE2}, the paralog of \textit{YIGE1}, regulates maize ear development and EL through auxin pathway. Knockout of \textit{YIGE2} causes a significant decrease of auxin level, IM length, floret number, EL, and grain yield. \textit{yige1 yige2} double mutants had even shorter IM and ears implying that these two genes redundantly regulate IM development and EL. The genes controlling auxin levels are differential expressed in \textit{yige1 yige2} double mutants, leading to lower auxin level. These results elucidated the critical role of \textit{YIGE2} and the redundancy between \textit{YIGE2} and \textit{YIGE1} in maize ear development, providing a new genetic resource for maize yield improvement.

Keywords: auxin, ear length, inflorescence meristem, maize, \textit{YIGE1}/\textit{YIGE2}.

INTRODUCTION

Maize (\textit{Zea mays} L.) is one of the most productive cereal crops and plays a critical role in supporting the growing world population (Hawkins et al., 2013). Longer ears afford more space for kernel development, resulting in a higher kernel number per row (KNPR) and consequently an elevated grain yield (Huo et al., 2016; Jia et al., 2020; Liu et al., 2021). Hundreds of ear length (EL) quantitative trait loci (QTL) have been mapped in maize (Li et al., 2018; Xiao et al., 2016; Yi et al., 2019), but only few causal genes have been cloned and studied in detail (Jia et al., 2020; Luo et al., 2022; Ning et al., 2021; Pei et al., 2022). A serine/threonine protein kinase encoded by \textit{KERNEL NUMBER ROW6} (\textit{KNR6}), phosphorylates an auxin regulating factor GTPase-activating protein (AGAP), and functions in auxin-dependent inflorescence development to increase grain yield by increasing floret number, EL, and KNPR (Jia et al., 2020). Another EL gene, \textit{ZmACO2}, which encodes 1-aminoacyclopropane-1-carboxylate oxidase2 (ACO2) is involved in ethylene biosynthesis. Knockout of \textit{ZmACO2} reduces ethylene production in developing ears, leading to longer ears and higher grain yield (Ning et al., 2021). Moreover, \textit{EAR APICAL DEGENERATION1} (\textit{EAD1}) encodes an aluminum-activated malate transporter and plays an essential role in regulating maize ear development and EL by transporting malate to the immature ear tip through xylem vessels (Pei et al., 2022). These studies highlight the complexity of the genetic basis underlying EL and grain yield, both of which are insufficiently understood.

The duplicate \textit{SBP}-box transcription factors genes \textit{Unbranched2} (\textit{UB2}), \textit{UB3}, and \textit{Tasselsheath4} (\textit{TSH4}), redundantly regulated multiple agronomic traits including plant height (PH), tassel branch number (TBN), and kernel row number (KRN). Mutations in either \textit{ub2}, \textit{ub3}, or \textit{tsh4} result in a modest reduction in PH, TBN, and an elevation in KRN. Disruption of two or more genes in the double and triple mutants leads to much severe decrease in PH, TBN, and increase in KRN (Chuck et al., 2014; Kong et al., 2022). The \textit{APETALA2} (\textit{AP2})-like genes \textit{indeterminate spikelet1} (\textit{ids1}) and \textit{sister of indeterminate spikelet1} (\textit{sid1}) play
multiple roles in maize inflorescence architecture. *sid1* and *ids1* single mutants were phenotypically normal of inflorescence architecture. *sid1 ids1* double mutants had fewer branches and often had bare rachis in place of the missing rows (Chuck et al., 1998, 2008). AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA)-related genes BARREN INFLORESCENCE1 (*BIF1*) and BARREN INFLORESCENCE4 (*BIF4*) regulate inflorescence axillary meristem initiation and determinacy. *bif1* and *bif4* mutants had reduced number of branches and spikelets, shortened ear with disorganized kernel rows. And these defects were more pronounced in *bif1 bif4* double mutants (Galli et al., 2015). These studies shed lights on the conservation and redundancy of the function of homologous genes.

Previously, we cloned another EL QTL, *YIGE1*, which positively regulates EL and grain yield by affecting inflorescence meristem (IM) length and floret number through sugar and auxin pathways (Luo et al., 2022). Here, we reported a new EL gene, *YIGE2*, a paralog of *YIGE1*. *YIGE2* knockout lines displayed a decreased EL, KNPR, and ear weight without changes of flowering or plant architecture traits. Loss-of-function mutation of *YIGE2* lead to significant decrease of auxin level. Double mutation of *yige1 yige2* dramatically enhanced the EL and the level of auxin. Our results showed that *YIGE2* plays a redundant role with *YIGE1* in regulating EL. The identification of *YIGE2* and its relationship with *YIGE1* expand our knowledge of the molecular mechanism of maize ear development.

RESULTS

**YIGE2 regulates maize EL**

*YIGE1*, encoding a cytoplasm-localized unknown protein, positively regulates maize EL and grain yield without affecting plant growth (Luo et al., 2022). Its homolog, *HCF243*, in *Arabidopsis* (*Arabidopsis thaliana*), was reported to encode a chloroplast localized protein and involved in D1 protein stability of the photosystem II complex, and its mutants had higher chlorophyll fluorescence with pale rosette leaves (Zhang et al., 2011). Therefore, *YIGE1* showed functional divergence in maize and *Arabidopsis* (Luo et al., 2022). *YIGE1* has one paralog, Zm00001d047617, which we named *YIGE2*. *YIGE2* shows 84.26% amino acid identity to *YIGE1* (Figure S1). The main sequence difference between them is an extra 262 amino acids at the N terminus of *YIGE2*. Despite this sequence difference, they exhibit similar expression patterns. Both of them are constitutively expressed and show highest expression level in developing ears (Figure 1A). In addition, *YIGE2* was also localized in the cytoplasm (Figure 1B; Figure S2), which is similar to the cellular localization of *YIGE1* (Luo et al., 2022). These results indicated the redundancy of *YIGE2* and *YIGE1* and also suggested that *YIGE2* may be involved in regulating ear development.

To determine the function of *YIGE2*, we used CRISPR-Cas9 to edit *YIGE2* and obtained three independent transgenic lines (*yige2-cr1*, *-cr2*, and *-cr3*). All three lines harbored one or more insertions/deletions that caused frame shift or early termination (Figure 1C). All three loss-of-function lines had a decrease in EL by -4.0–10.2%, KNPR by -6.4–11.0%, and ear weight by -11.7–22.9% when comparing with their corresponding wild-type (WT) siblings across multiple environments (Figure 1D–G; Figure S3). Moreover, we measured a set of agronomic traits, including flowering time, PH, ear height, ear leaf length and width, tassel main axis length, TBN, and KRN. Compared to WT, *yige2* knockout lines had no significant difference in flowering time, KRN, and plant architecture traits (Figures S4–S6). These results confirmed the function of *YIGE2* in regulating EL, KNPR, and grain yield without the penalty of other agronomic traits, and also highlighted the great potential of *YIGE2* for maize improvement.

**YIGE2 regulates IM size and florets number**

During the reproductive stage, the ear IM forms spikelet meristems that develop into spikelet and floral meristems (Vollbrecht & Schmidt, 2009). A larger IM provides more space for floral meristem development, contributing to EL through a higher floret number (Jia et al., 2020; Luo et al., 2022). To investigate the mechanism of *YIGE2* in regulating ear development, we used scanning electron microscope to visualize the developing inflorescence. After measuring the length of IM in 3-4 mm developing ears and counting the floret number before pollination, we found that *yige2-cr3* knockout lines had a significant shorter IMs, reduced floret number per row, and shorter EL when comparing with WT (Figure 2). There was no significant difference of the number of kernels developed from florets after pollination between *yige2-cr3* and WT (Figures 1E and 2C). These findings indicate that *YIGE2* controls ear development through the regulation of IM and floret number.

**YIGE2 plays a redundant role with **

To further elucidate the genetic relationship between *YIGE2* and *YIGE1*, we generated double-mutants through crossing *yige2* with *yige1*. In the F2, the mutants segregated according to Mendel’s laws of independent assortment and segregation (Figure S7), indicating that these two genes are inherited independently. As expected, the double mutants had a more pronounced phenotype with an even shorter IM comparing with either *yige2* or *yige1* (Figure 3A,B). Consequently, much shorter EL and less KNPR were observed in double mutants than that of single mutants (Figure 3C–E; Figure S8). In addition, there was no significant difference in KRN between WT, *yige1*, *yige2*, and *yige1 yige2* (Figure 3F). In summary, these results confirmed that...
YIGE2 had redundant role with YIGE1 in regulating maize ear development.

**YIGE2 and YIGE1 are involved in an auxin signaling pathway**

To identify the regulatory network in which YIGE2 and YIGE1 were involved, we conducted RNA-seq using 2 mm developing ears from WT, yige1, yige2, and yige1 yige2. Compared to WT, we identified 559 differentially expressed genes (DEGs) in yige2 with 247 genes upregulated and 312 genes downregulated (Figure S9a). In yige1, 757 DEGs were detected with 376 genes upregulated and 381 genes downregulated (Figure S9b). Additionally, we detected 720 DEGs in yige1 yige2 with 315 genes upregulated and 40 genes downregulated.
genes downregulated (Figure 4A). After performing gene ontology (GO) analysis, 26 GO terms were enriched in both single mutants and double mutant, including response to hormone, response to temperature stimulus, and response to abiotic stimulus (Figure 4B; Figure S9c–e). Hormones, especially auxin, play a crucial role in maize ear development (Gallavotti, 2013; Galli et al., 2015; Jia et al., 2020; Luo et al., 2022; McSteen et al., 2007; Ross et al., 2001; Zhao, 2010). Therefore, we primarily focused on the enriched GO item of response to hormone and identified several auxin-related genes, including Zm00001d048709 encoding IAA biosynthesis I, Zm00001d043660 encoding auxin efflux carrier family protein, Zm00001d010697 encoding auxin-responsive GH3 family protein, and Zm00001d032422 encoding auxin associated family protein, which are differentially expressed in yige1 yige2 (Figure 4C).

To further explore the connection between YIGE2/YIGE1 and auxin, we next measured the auxin level via LC–MS/MS in 2–5 mm developing ears of WT, yige1, yige2, and yige1 yige2. There were no significant differences in the levels of 3-indoleacetonitrile, indole-3-carboxylic acid, and methyl indole-3-acetate when comparing mutants with WT (Figure S10). However, we observed significant differences in the levels of indole-3-acetic acid (IAA), indole-3-acetyl-l-aspartic acid (IAA-Asp), indole-3-acetyl glutamic acid, 2-oxindole-3-acetic acid (OxIAA), L-tryptophan, indole, and its precursor substance tryptamine (Figure 4D; Figure S10). Moreover, the reductions of IAA, IAA-Asp, and OxIAA content in yige1 yige2 were greater.

Figure 2. YIGE2 regulates inflorescence meristem and floret production. (A) Micrograph of 3–4 mm immature apical ears of wild type and YIGE2 knockout lines. Yellow lines represent inflorescence meristem length. (B–E) Inflorescence meristem (IM) length (B), floret number per row (C) and ear length before pollination (D, E) of WT and yige2-cr3. Data in (B–D) are shown as the mean ± SD. The significant differences are determined by one-way ANOVA analysis. Each blue dot indicates phenotype value of each sample in (B–D). ***P < 0.001. Scale bar = 200 μm in (A) and 2 cm in (E).
relative to either *yige1* or *yige2* (Figure 4D). Taken together, our results suggested that both *YIGE2* and *YIGE1* participate in the regulation of auxin content (Figure 4E).

**DISCUSSION**

Ear length plays an important role in maize grain yield (Xu et al., 2018; Zhou et al., 2018). Previous studies have shown that EL was affected by IM size, florets production, and floral fertility (Jia et al., 2020; Luo et al., 2022; Ning et al., 2021; Pei et al., 2022). *KNR6* and *YIGE1* enhanced EL and grain yield by raising IM size and floret production without altering floral fertility (Jia et al., 2020; Luo et al., 2022). By contrast, *ZmACO2* loss of function lines showed increased EL by promoting IM with more florets and enhanced floret fertility (Ning et al., 2021). Moreover, dysfunction of *EAD1* resulted in apical IM degeneration with SPMs/SMs collapsing extending and deteriorating in ≥20 mm inflorescences, leading to the abortion of florets and ultimately a shorter EL (Pei et al., 2022). These studies highlighted the complexity of genetic bases and molecular mechanism of EL. In our current study, *YIGE2* knockout lines were found to have a shorter IM length and EL, lower FNPR, and similar floret fertility (Figures 1 and 2). In addition, *yige1 yige2* showed a more severe phenotype (Figure 3), suggesting that *YIGE2* plays a redundant role with *YIGE1* in regulation of maize.*YIGE2/1* are expressed in multiple tissues, yet the knockout of *yige1* and *yige2* appears to specifically affect ear phenotypes. One plausible explanation for this observation is that *YIGE2/1* are expressed at higher levels in the developing ear compared to other tissues, which could contribute to its predominant effect on ear phenotypes upon knockout. Moreover, *YIGE2/1*, being a cytoplasmic.

protein, may not directly regulate gene expression at the transcriptional level. Instead, it is conceivable that YIGE2/1 interacts with other proteins or forms complexes that play crucial roles in ear development. These interactions could involve signaling pathways, protein–protein interactions, or posttranslational modifications that specific to the ear tissue. To explore the interaction proteins with YIGE2/1, we identified 13 potential interaction proteins with YIGE2/1 based on the published data (Han et al., 2023), and three was further selected for luciferase complementation assay in tobacco (Table S1). Unfortunately, none positive interaction was detected (Supplementary Fig. 11). Therefore, investigating the downstream targets or interacting partners of YIGE2/1 in the context of ear development will require more considerable future research effort.

Maize was an ancient tetraploidy and this polyploidy resulted in there are two duplicates (maize 1 and maize 2 subgenomes) of the current diploid maize genome (Salse et al., 2008; Wei et al., 2007). YIGE1 is located in chromosome 1 and belongs to maize1 subgenome, while YIGE2 is located in chromosome 9 and belongs to maize2 subgenome in modern maize (Schnable et al., 2011). Moreover, segments of YIGE1 and YIGE2 in maize blasted to the same sorghum chromosome1 (Schnable et al., 2011). These results indicated that the mechanisms of retaining the two genes is the ancient polyploidy. Moreover, the functional

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**Figure 4.** YIGE1 and YIGE2 affect EL through regulating auxin signaling.  
(A) Differentially expressed genes between WT and yige1 yige2. Each dot represents a gene. Blue and red dots represented the downregulated and upregulated genes, respectively.  
(B) Significantly enriched Gene Ontology items (false discovery rate <0.05) shared by single and double mutants.  
(C) Auxin-related differentially expressed genes.  
(D) Quantification of auxin in the developing ears of WT, yige1, yige2 and yige1 yige2. Data are shown as the mean ± SD. The significant differences are determined by multiple comparisons and a,b,c,d indicate significant differences (P < 0.05). Each blue dots represent the phenotype valve of each sample in (D).  
(E) A proposed model to illustrate the regulatory pathway in which YIGE2 and YIGE1 are involved.
redundancy of YIGE1 and YIGE2 can be protective, ensuring that loss of function in one gene does not result in a loss of the essential gene function in regulating maize ear development.

Moreover, we found that GO items of auxin pathway were significantly enriched in yige1, yige2, and yige1 yige2 but not sugar pathway related GO items (Figure 4B; Figure S9). This is inconsistent with the findings in YIGE1 overexpression materials (Luo et al., 2022). It may be attributed to the full-length coding sequence of YIGE1 driven by the ubiquitin promoter in YIGE1 overexpression lines, leading to some other effects associated with ectopic expression. Auxin plays a critical role in regulation of florets production on maize ear (Carraro et al., 2006; Jia et al., 2020; Luo et al., 2022; McSteen et al., 2007; Phillips et al., 2011), which is consistent with our observation that decreased auxin contents in yige1, yige2 result in fewer floret number, shorter EL, and lower grain yield (Figures 2–4).

YIGE1 underwent continuous selection, and the favorable allele was enriched during maize domestication and improvement (Luo et al., 2022). However, we did not find the evidence that YIGE2 was under selection during maize domestication and improvement (Chen et al., 2022). We try to find the possible variations in YIGE2 correlated to EL in 513 association mapping population and identified 57 single nucleotide polymorphisms (SNPs) in 1 kb promoter region and 88 SNPs in gene body region of YIGE2 (Chen et al., 2022). However, no SNPs show significant correlation with EL. In addition, overexpression of YIGE1 resulted in a higher EL and elevated grain yield. YIGE2 and YIGE1 exhibit conservation in regulation of maize ear development and grain yield (Figures 1–3). We speculated that higher expression level of YIGE2 may increase EL and grain yield, similar with YIGE1. Therefore, it is promising to obtain longer ears and higher grain yield through enhancing the expression level of YIGE2 via editing the promoter of YIGE2 by CRISPR/-Cas9 technology or overexpressing YIGE2. Moreover, further studies are required to reveal more precise details concerning the function of YIGE1 and YIGE2 as these will be helpful in understanding maize ear development as well as facilitating the genetic improvement of grain yield.

MATERIALS AND METHODS

Vector construction, genetic transformation, and identification of the transgenic maize

CRISPR/Cas9 was used to induce mutations in the YIGE2 (Zm00001d047817) exon region. Single-guide RNAs (sgRNAs) were designed based on the B73 reference genome using CRISPR-P webtool (Liu et al., 2017) (http://crispr.hzau.edu.cn/CRISPR2). Four sgRNAs were selected, synthesized, and cloned into the pCPB2mUbi-hspCas9 binary vector following the manufacturer’s recommended protocols (Li et al., 2017). Subsequently, the CRISPR-Cas9 plasmids were transformed into the inbred line KN5858 via Agrobacterium-mediated transformation at Weimi Biotechnology in Jilin, China (Luo et al., 2020). Genomic editing of YIGE2 was screened by PCR amplification and Sanger sequencing of the target regions. Cas9-negative edited plants underwent selfing for two generations before further phenotypic scoring. The edited lines were cultivated in Jilin, China (43.5° N, 124.82° E), and Hubei (Wuhan; 30.58° N, 114.31° E), China. The plants were grown in 2.5 m rows, spaced 0.5 m apart, with 11 individuals in each row. The sgRNAs and primers used for genotype of YIGE2 are provided in Table S2.

Double mutant analysis

The yige1 yige2 double mutants were generated by crossing the YIGE2-c3 knockout lines to YIGE1 knockout lines. For genotyping, leaf tissue was collected from 4-week-old plants, and DNA was extracted according to a modified CTAB protocol. PCR was carried out to genotype the plants using primers CR8490-F (5’ ATGATGGGAGTGGAGG 3’ ) and CR8490-R (5’ TGGTCCTCGG AGACTTGTG 3’ ) for YIGE1, and using primers HZY7-F (5’ GGCCG ATGCTACACAATTCAC 3’ ) and HZY7-R (5’ CACCTCCACTCCCTC TACCA 3’ ) for YIGE2, respectively. The yige1 yige2 double mutants were easily identified based on the genotype. For analysis of EL, KNPR, and ear weight, >30 plants of each genetic class were analyzed.

Phenotype measurement and data analysis

By 40 days of plant growth, wild type, yige1, yige2, and yige1 yige2 double mutants –4 mm developing ear were collected and taken pictures using a stereomicroscope (Olympus; SZX10; Tokyo, Japan). Next, the size of IM was measured using ImageJ (Schindelin et al., 2015) and analyzed by Excell 2020. Moreover, we collected maize ears before and after pollination from WT and yige2 knockout lines, and counted KNPR and measured EL with ruler. Progeny tests were performed by the Student’s test.

Subcellular localization of YIGE2

The full-length YIGE2 coding sequence was amplified from B73 cDNA and cloned downstream of the CaMV35S promoter in the pCAMBIA1302 vector that carried GFP. A pCambia1302-35S:YIGE2-GFP construct was introduced into maize protoplasts as described previously (Yoo et al., 2007). The GFP signals were detected using a FV1200 Laser Scanning Microscope (Olympus Corporation). The primers of cloning full-length YIGE2 coding sequence are listed in Table S2.

RNA-seq

Total RNA was extracted from ~2 mm developing ears of WT, yige1, yige2, yige1 yige2 double mutants using a Quick RNA Isolation Kit (Huayueyang Biotechnology Co., Ltd., Beijing, China). For RNA-seq, three biological replicates were performed for each sample. The RNA libraries were prepared using commercial Illumina library preparation kits (TruSeq Stranded mRNA LT-SetA. RS-122-1201) and subsequently sequenced following the HiSeq X-Ten protocols. Trimmomatic (v0.36) was employed to filter out lower-quality sequencing reads (Bolger et al., 2014). RSEM-1.3.0 was utilized for aligning RNA-seq reads to the B73 v4 reference genome, and gene expression levels were estimated using default parameters (Li & Dewey, 2011).

Detection the levels of auxin in developing ear

Fresh immature ears (2.5–4 mm) from WT, yige1, yige2 and yige1 yige2 double mutants with at least three biological replicates were
obtained and saved in liquid nitrogen. Then auxin contents were quantified by MetWare (http://www.metware.cn/). Approximately 50 mg of powder was dissolved in 1 ml mixture of methanol, water and formic acid (15:4:1, v/v/v), and 10 μl of the internal standard mixture solution (100 ng ml⁻¹) was added to the extract as internal standards (IS) for the quantification. The resulting mixture was vortexed for 10 min at 4°C. Following centrifugation at 18 400 g at 4°C, the supernatant was carefully transferred to a clean plastic microtube and subjected to evaporation until dryness. The dried samples were reconstituted in 100 μl of 80% methanol (v/v), filtered through a 0.22 μm membrane and subsequently transferred to sample vials for subsequent LC-MS/MS analysis (Floková et al., 2014; Li et al., 2016; Simura et al., 2018) in an UPLC-ESI-MS/MS system consisting of UPLC, ExionLQ™ AD (https://sciex.com.cn/) and MS, Applied Biosystems 6500 Triple Quadrupole (https://sciex.com.cn/). The auxin levels were assessed by comparing the response of the IS added during extraction to the developing ear sample components.

AUTHOR CONTRIBUTIONS

YLuo and JY designed and supervised this study. YLuo, YLi and HL performed the field trials, validation of gene function and gene expression analysis. CJ constructed the transgenic vector. YLuo, JY, AF, JL, and DJ wrote and revised the manuscript. All the authors read and approved the paper.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

SUPPLEMENTARY INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Amino acid sequence alignment of YIGE1 and YIGE2.

Figure S2. The subcellular localization of YIGE2.

Figure S3. Transgenic validation of YIGE2 in JILin, China.

Figure S4. Flowering traits and plant architecture of wild type and yige2-cr1 knockout lines.

Figure S5. Flowering traits and plant architecture of wild type and yige2-cr2 knockout lines.

Figure S6. Comparison of kernel row number between wild type and yige2-cr3 knockout lines.

Figure S7. Selecting double-mutant materials in the F₂ segregating population.

Figure S8. Comparison of ear length between wild type, yige1, yige2, and yige1 yige2 double mutant.

Figure S9. RNA-seq analysis in WT, yige1, yige2, and yige1 yige2 double mutants.

Figure S10. Auxin content in the developing ear of WT, yige1, yige2, and yige1 yige2 double mutants.

Figure S11. Analysis of protein interaction between YIGE2/1 and Zm00001d047757, Zm00001d049294, and Zm000001d049346.

Table S1. The potential interaction proteins of YIGE2/1.

Table S2. Primers are used in the experiment.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The RNA-seq data from this study have been deposited in CNGBdb (https://db.cngb.org/), and under project accession code CNP0005548. And the full genomic sequence of YIGE2 can be found in GenBank (accession no. Bankit2819411 Seq1PP693109).

DATA AVAILABILITY STATEMENT

The RNA-seq data from this study have been deposited in CNGBdb (https://db.cngb.org/), and under project accession code CNP0005548. And the full genomic sequence of YIGE2 can be found in GenBank (accession no. Bankit2819411 Seq1PP693109).

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