A High-Quality, Chromosome-Level Genome Provides Insights Into Determinate Flowering Time and Color of Cotton Rose (*Hibiscus mutabilis*)

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**Specialty section:** This article was submitted to Plant Bioinformatics, a section of the journal Frontiers in Plant Science

Received: 19 November 2021  Accepted: 20 January 2022  Published: 14 February 2022

**Citation:** Yang Y, Liu X, Shi X, Ma J, Zeng X, Zhu Z, Li F, Zhou M, Guo X and Liu X (2022) A High-Quality, Chromosome-Level Genome Provides Insights Into Determinate Flowering Time and Color of Cotton Rose (*Hibiscus mutabilis*). Front. Plant Sci. 13:818206. doi: 10.3389/fpls.2022.818206

*Hibiscus mutabilis* (cotton rose) is a deciduous shrub or small tree of the Malvaceae family. Here, we report a chromosome-scale assembly of the *H. mutabilis* genome based on a combination of single-molecule sequencing and Hi-C technology. We obtained an optimized assembly of 2.68 Gb with a scaffold N50 length of 54.7 Mb. An integrated strategy of homology-based, de novo, and transcriptome-based gene predictions identified 118,222 protein-coding genes. Repetitive DNA sequences made up 58.55% of the genome, and LTR retrotransposons were the most common repetitive sequence type, accounting for 53.15% of the genome. Through the use of Hi-C data, we constructed a chromosome-scale assembly in which Nanopore scaffolds were assembled into 46 pseudomolecule sequences. We identified important genes involved in anthocyanin biosynthesis and documented copy number variation in floral regulators. Phylogenetic analysis indicated that *H. mutabilis* was closely related to *H. syriacus*, from which it diverged approximately 15.3 million years ago. The availability of cotton rose genome data increases our understanding of the species’ genetic evolution and will support further biological research and breeding in cotton rose, as well as other Malvaceae species.

**Keywords:** *Hibiscus mutabilis*, genome, Hi-C, phylogenetic affiliation, floral regulators

**INTRODUCTION**

*Hibiscus mutabilis* is one of the most popular tree species in the Malvaceae family, which includes species such as *Gossypium raimondii* and *Hibiscus syriacus* (Rose of Sharon). Some members of the Malvaceae have relatively high economic value. For example, cotton is the largest source of natural textile fibers in the world, and over 90% of its annual fiber production comes from allotetraploid cotton (*G. hirsutum* and *G. barbadense*) (Wang et al., 2019). Additionally, many Malvaceae species are used as ornamentals because of their flowers. *H. syriacus* is an important horticultural species whose attractive white, pink, red, lavender, or purple flowers are displayed over a long bloom period, although individual flowers last only a day in the landscape (Kim et al., 2016). This study of *H. mutabilis* (2n = 92) (Li et al., 2015) focuses primarily on its ornamental characters, including...
its flower colors, long bloom time, and floral development and morphogenesis. In addition to its ornamental value, *H. mutabilis* is also an ingredient in local herbal remedies. It is thought to cool the blood, relieve toxins, reduce swelling, and alleviate pain, and it has long been used in the treatment of ulcers, swelling, herpes zoster, scalding, bruises, etc. (Liu et al., 2015). The complete genome sequence of an organism provides a large amount of information for subsequent biological studies (Yang et al., 2005). The *H. mutabilis* genome sequencing project is therefore extremely valuable for breeding, comparative genomics research, and other activities.

*H. mutabilis* has been cultivated for more than 2,000 years south of the Yangtze River; it is also the city flower of Chengdu and has great significance for the city. Commonly used as an ornamental species, its attractive purple, red, pink, or white flowers are displayed over a long bloom period (3–4 months or more), although its individual flowers last for less than 48 h (Figure 1). During flower development, the floral color of some varieties shows little change, but that of other varieties undergoes a marked change from white to pink within a single day. This interesting dynamic phenomenon can be seen in the cultivars 'Drunk girl' and 'Bairihuacai' and occurs during the process of individual flower development, unlike the color differences found in distinct cultivars of chrysanthemum (Ohmiya et al., 2006), *Narcissus pseudonarcissus* (Li et al., 2018), or *Brassica napus* (Zhang et al., 2015).

The development of genomic resources and molecular breeding technologies holds promise for targeted character improvement of *H. mutabilis* in the near future. Recently, a 1.75 Gb draft genome of *H. syriacus* was assembled, and a chromosome-scale genome of *H. cannabinus* was published in 2020 (Zhang et al., 2020). Many breeding systems and novel varieties have been produced using traditional methods to meet horticultural requirements, but a completed genomic sequence will accelerate the breeding of cotton rose.

The many flower colors of cotton rose give it high ornamental value and reflect the complexity of the underlying flavonoid metabolic pathway. One endpoint of flavonoid biosynthesis is the production of anthocyanins, pigments that produce the colors of many flowers, fruits, and other plant tissues (Koes et al., 1994). Chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), and flavonoid 3′-hydroxylase (F3′H) all function in the synthesis of anthocyanins and anthocyanidins, their aglycone counterparts. CHS represents the first committed step in the flavonoid pathway (Meer et al., 1993). The second step is performed by CHI, which acts on the yellow naringenin chalcone product of CHS, catalyzing its isomerization to the colorless flavanone naringenin (Moustafa and Wong, 1967). Dihydroflavonols are subsequently reduced to leucoanthocyanidins by DFR (Durbin et al., 2004). ANS catalyzes the formation of cyanidin from leucoanthocyanidins and is the penultimate step in the biosynthesis of the anthocyanin class of flavonoids (Figure 2). Despite recent progress in understanding *H. mutabilis* anthocyanidin biosynthesis, the lack of a genome sequence has hampered efforts to elucidate the molecular and genetic determinants of this trait, which underlies the dynamic phenomenon of flower color development. Genome and transcriptome sequences are needed in order to fully analyze the molecular mechanisms of anthocyanidin biosynthesis.

In the present study, we generated a reference genome for *H. mutabilis* using a combination of single-molecule sequencing and Hi-C technology. We identified functional genes involved in the biosynthesis of anthocyanins based on homology searches and functional annotations. We also investigated copy number variation in floral regulators among multiple species to gain insight into the evolution of flowering phenotypes in *H. mutabilis*. The genomic resources developed here will be useful for further experimentation, cultivation, and breeding of *H. mutabilis* and other Malvaceae species.

**MATERIALS AND METHODS**

**Plant Materials and Whole-Genome Sequencing**

The *H. mutabilis* material sequenced in this study was the stably heritable single-petal white color cultivars, which is cultivated in the nursery of the Chengdu Botanical Garden (CDBG), Sichuan, China. The breeding system of *H. mutabilis* belongs to allogamy. Seeds of 'single-petal white' were collected in the laboratory of the CDBG. Young leaves (∼3 cm width) were harvested to extract high-quality DNA for Illumina and Oxford Nanopore Technology (ONT) sequencing. For transcriptome sequencing, petals were manually collected from three color cultivars ('single-petal white,' 'single-petal pink,' and 'Purple silk') and at three stages of color development in 'Drunk girl' (white, blended white and pink, and fully pink). Flowers at the same stage from individual *H. mutabilis* plants were pooled and divided into three samples. These samples were immediately frozen in liquid nitrogen and then used for RNA sequencing.

High-quality *H. mutabilis* genomic DNA was extracted from young leaves with a DNA secure Plant Kit (TIANGEN, China) and used to construct long-read libraries for the ONT platform. Libraries were prepared following the ONT 1D Genomic DNA by Ligation (Kit 9 chemistry)-PromethION protocol and sequenced using the PromethION protocol. In addition, high-quality DNA was broken into random fragments, and an Illumina paired-end library was constructed with an insert size of 350 bp and sequenced using the Illumina HiSeq X Ten platform.

For Hi-C sequencing, leaves were fixed with 1% formaldehyde solution in MS buffer (10 mM potassium phosphate, pH 7.0; 50 mM NaCl; 0.1 M sucrose) at room temperature for 30 min in a vacuum. After fixation, the leaves were incubated at room temperature for 5 min under a vacuum in MC buffer with 0.15M glycine. Approximately 2 g of fixed tissue was homogenized with liquid nitrogen, resuspended in nuclei isolation buffer, and filtered with a 40-nm cell strainer. The procedures for enriching nuclei from flow through and subsequent denaturation were performed according to a 3C protocol. The chromatin extraction procedures were similar to those described previously. In brief,
**FIGURE 1** | *Hibiscus mutabilis* floral morphology. (A) Dynamic change in the flower color of *H. mutabilis* f. *mutabilis* ‘Drunk girl’ from white to pink. (B) A variety of colors found in different color cultivars of *H. mutabilis*, and from left to right in turn are *H. mutabilis* ‘Single-Petal Pink,’ *H. mutabilis* ‘Single-Petal White’ and *H. mutabilis* ‘Purple silk.’

**FIGURE 2** | An abbreviated diagram of the flavonoid pathway that produces anthocyanins. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, 3-O-glucosyl transferase.
concatenated using solar (Yu et al., 2007). For each blast hit, E
with an
Protein sequences were aligned to the genome using TBLASTN
T. cacao
and
H. mutabilis
database. Overlapping TEs that belonged to the same type of
level, RepeatProteinMask (Tarailo-Graovac and Chen, 2009) was
using Tandem Repeats Finder (Benson, 1999). At the protein
de novo
2009) was used for DNA-level identification with Repbase
TE library. RepeatMasker (Tarailo-Graovac and Chen,
Supplementary Table 4
(Tarailo-Graovac and Chen, 2009), Piler (Edgar and Myers,
DNA and protein levels. We used RepeatModeler, RepeatScout
Genome Annotation
Supplementary Table 5
(Simão et al., 2015; and BUSCO (Benchmarking Universal Single-Copy Orthologs)
(Neves et al., 2015) database. rRNA genes were identified using BLASTN
against the KEGG (Kanehisa and Goto, 2006) database with an
obtained from the corresponding InterPro descriptions. Putative
pathway assignments for each gene were obtained by blasting against the KEGG (Kanehisa and Goto, 2006) database with an
E-value cutoff of 1 × 10^-5.

tRNA genes were predicted by tRNAscan-SE (Lowe and Eddy, 1997), and miRNA and snRNA fragments were identified using Infernal (Nawrocki et al., 2009) with the Pfam (Griffiths-Jones et al., 2005) database. rRNA genes were identified using BLASTN
(E-value ≤ 1 × 10^-10) against the plant tRNA database.

Genome Evolution Analysis
First, nucleotide and protein data from nine species (A. trichopoda, A. thaliana, B. ceiba, C. capsularis, D. zibethinus, G. raimondii, H. syriacus, R. chinensis, and T. cacao) were downloaded from Ensembl, NCBI, and JGI. The longest transcript was selected from the alternatively spliced transcripts of each gene, and genes with ≤ 50 amino acids were removed. Nucleotide and protein data from H. mutabilis and the other
nine angiosperms were clustered into orthologous groups using
BLASTp and OrthoMCL v2.0.9, and an MCL inflation of 1.5 was used as the cluster granularity setting (Li et al., 2003). A phylogenetic tree was constructed using shared single-copy orthologs. Protein sequences of the orthologs were aligned using MUSCLE (Edgar, 2004), and the protein alignments were transformed to CDS alignments. We then concatenated the CDS alignments into a “supermatrix” from which the phylogenetic tree was constructed using the maximum likelihood (ML) TREE algorithm in RAxML v8.1.13 (Stamatakis, 2006) with the best-scoring protein substitution model (GTR+gamma)
and 1,000 bootstrap replicates. The MCMCtree program in the PAML package (Yang, 1997) was used to estimate divergence times among the ten species. Three fossil calibration points were used for restraining the age of the nodes: 23–48 Mya (Million years ago) for the MRCA of \textit{T. cacao–G. raimondii}, 65–107 Mya for the MRCA of \textit{G. raimondii–A. thaliana} (Wang et al., 2012), and 103–109 Mya for the MRCA of Malvales–Rosales (Wikström et al., 2001). CAFE was used to identify expansions and contractions within orthologous gene families by comparing cluster size differences between the ancestor and other species (De Bie et al., 2006). To estimate the synonymous substitutions per synonymous site (Ks), all paralogous gene pairs were analyzed with the ML method in PAML (Yang, 1997). MCscan (Tang et al., 2008) was used to analyze genome collinearity in \textit{H. mutabilis}.

**Identification of Nucleotide-Binding Site-Encoding Genes**

To identify NBS-encoding genes, representative genes from each plant genome were screened using a raw Hidden Markov Model (HMM3.0) (Marchin et al., 2005) to search for the Pfam NBS family PF00931 domain with an E-value cut-off of 1.0. All putative NBS protein sequences were analyzed and manually curated based on a TBLASTN search against known R gene sequences in GenBank. To further identify TIR homologs and sequences that encoded CC and LRR motifs, candidate NBS-LRR protein sequences were characterized using SMART (Schultz et al., 1998), the Pfam database (Finn et al., 2013), and the COILS program (Lupas et al., 1991) with a threshold of 0.9 to specifically detect the CC domain.

**Transcriptome Sequencing**

For analysis of flowering gene(s), petals were manually collected from three color cultivars at the same time (2–3 p.m.) and at three stages of color development in ‘Drunk girl’ (white at the 9 a.m., blended white and pink at the 12 a.m., and fully pink at 6 p.m.) and these samples were immediately frozen in liquid nitrogen and then used for RNA sequencing, and total RNA was extracted using an RNAprep Pure Plant Kit (TIANGEN, China). The quality and quantity of the RNA samples were evaluated using a NanoPhotometer (Implen, CA, United States), a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, United States), and an Agilent 2,100 Bioanalyzer (Agilent Technologies, United States). All RNA samples with integrity values greater than 7.0 were used for cDNA library construction and sequencing. The cDNA libraries were prepared using the NEB Next Ultra RNA Library Prep Kit (E7350L, NEB, United States), and 150-bp paired-end sequencing was performed on the Illumina NovaSeq 6000 platform (Illumina, CA, United States).

**RESULTS**

**Genome Sequencing and Assembly**

We assembled the \textit{H. mutabilis} genome using a combination of Illumina HiSeq X Ten and Oxford Nanopore PromethION sequencing. We generated 315.22 Gb (104-fold coverage) of raw 150-bp paired-end Illumina reads and 469.91 Gb (155-fold coverage) of raw Nanopore reads. The genome size was estimated to be 3032.98 Mb based on the 17-mer depth distribution (Supplementary Table 1 and Supplementary Figure 1). Nanopore long reads were assembled into contigs and scaffolds using wtdbg2 v2.5.13 resulting in a final assembly of 2.68 Gb with 5,464 contigs and a contig N50 of 2.22 Mb (Supplementary Table 2). Its GC percentage was 35.36%, similar to that of the \textit{H. syriacus} genome (34.04%). In total, 363.5 Gb of clean reads were obtained from Hi-C sequencing (over 121-fold coverage). We used these data to construct chromosome-scale scaffolds, resulting in a total of 5,598 contigs, with a scaffold N50 of 54.70 Mb and a total length of 2,676,237,573 bp (Supplementary Table 10 and Figure 3A).

Next, The clustering of contig by hierarchical clustering of the Hi-C data was performed. Hi-C linkage was used as a criterion to measure the degree of tightness of the association between different contigs by standardizing the digestion sites of \textit{DpnII} on the genome sketch. The contigs were assembled into 46 pseudo-chromosomes using LACHESIS package tools. The Illumina paired-end reads were mapped to the assembled genome to assess assembly accuracy, resulting in a 98.81% mapping rate (Supplementary Table 3 and Figure 3B). The genome assembly captured 96.77% of the core eukaryotic genes from CEGMA18 (Supplementary Table 4) and 92.6% of the Embryophyta OrthoDB gene set in BUSCO19 (Supplementary Table 5), indicating a high level of completeness.

**Genome Annotation**

We identified 1.56 Gb of non-redundant repetitive elements, representing approximately 55.85% of the \textit{H. mutabilis} genome assembly. Because long terminal repeat retrotransposons (LTR-RTs) typically make a significant contribution to large genome size (Zhao and Ma, 2013), we estimated LTR-RT insertion time in \textit{H. mutabilis}. We identified a round of LTR-RT burst approximately 2.5 million years ago (Mya), especially for the Ty3/Gypsy-del and Ty1/Copia Retrofit families (Supplementary Table 6). The transposable elements (TEs) were primarily long terminal repeats (LTRs), which accounted for approximately 53% of the genome (Supplementary Figure 2).

We used \textit{de novo} and homology-based gene prediction approaches and combined their results to annotate 118,222 protein-coding genes in the \textit{H. mutabilis} genome. The average transcript length was 2,466.97 bp, with an average of 4.53 exons per gene and an average exon length of 218.86 bp. Compared with other model plants and Malvaceae species, the \textit{H. mutabilis} genome contained a larger number of genes: \textit{H. syriacus} (82,827 genes), \textit{Arabidopsis thaliana} (26,869), \textit{Theobroma cacao} (29,144), \textit{G. raimondii} (35,526), \textit{Corchorus capsularis} (29,356), \textit{Herrania umbratica} (29,262), and \textit{Durio zibethinus} (63,819) (Supplementary Table 7).

In addition to RNA-coding genes, we also identified 827 mature microRNAs (miRNAs), 3,604 transfer RNAs (tRNAs), 3,423 ribosomal RNAs (rRNAs), and 9,370 small nuclear RNAs (snRNAs) in the \textit{H. mutabilis} genome (Supplementary Table 11).
FIGURE 3 | (A) Chromosomal features of *H. mutabilis*. (a) Gene density; (b) Repeat density; (c) AT content; (d) Syntonic blocks. (B) Hi-C map of the *H. mutabilis* genome showing genome-wide all-by-all interactions. The map shows a high resolution of individual chromosomes that were scaffolded and assembled independently. Color intensity indicates the frequency of Hi-C interaction links from low (yellow) to high (red).
The probable functions of the predicted genes were assessed by searching against public databases, including Swiss-Prot, NR, InterPro, and KEGG of 118,222 predicted genes in the *H. mutabilis* genome, 113,821 (96.3%) were assigned potential functions as a result of these database searches (Supplementary Table 8).

**Genome Evolution**

Although morphological investigations have placed *H. mutabilis* in the Malvaceae family, there is still no phylogenomic analysis of its evolutionary position within the family based on whole-genome data. Here, we compared the *H. mutabilis* genome with the genome sequences of nine other angiosperm plants (*H. syriacus*, *A. thaliana*, *Rosa chinensis*, *Bombax ceiba*, *Amborella trichopoda*, *G. raimondii*, *T. cacao*, and *C. capsularis*). Orthologous protein groups were identified within the genomes, yielding a total of 30,208 gene families and 198 single-copy orthologs across ten species. There were 2,781 gene families specific to *H. mutabilis*, and 10,593 gene families were shared among all species investigated (Figure 4). We detected 4,558

![Figure 4](https://example.com/figure4.png)

**FIGURE 4** | Comparative genomics and evolution of gene numbers. (A) The number of genes in cluster of ten plant species, showing a high gene number in *H. mutabilis* compared with the model plant *A. thaliana* and other angiosperm species. The number of multiple-copy paralogs is high in *H. mutabilis*. (B) Venn diagram showing the numbers of shared gene families among *H. mutabilis* (Hmu), *A. thaliana* (Ath), *H. syriacus* (Hsy), *R. chinensis* (Rich), and *G. raimondii* (Gra). (C) Phylogenetic relationships of NBS genes in *H. mutabilis*.
gene families expansion when *H. mutabilis* and *H. syriacus* have diverged (Figure 5C).

We constructed a phylogenetic tree based on single-copy genes using PAML and estimated the divergence times among the 10 species. The Malvaceae family appeared to have diverged from a Tiliaceae–Malvaceae most recent common ancestor (MRCA) approximately 45.8 (34.8–55.6) Mya, and the *Hibiscus*-*Gossypium* divergence was estimated at 23.1 (16.0–30.5) Mya.
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TABLE 1 | Copy numbers of genes encoding flowering time regulators in five plant species.

| Gene | Arabidopsis locus | Copy number |
|------|------------------|-------------|
|      |                  | H. mutabilis | H. syriacus | A. trichopoda | T. cacao | G. raimondii |
| CO   | AT5G15840        | 8           | 9           | 7             | 3       | 2           |
| ELF4 | AT2G40080        | 9           | 12          | 2             | 1       | 5           |
| FCA  | AT4G16280        | 4           | 0           | 1             | 2       | 1           |
| FKE1 | AT1G68050        | 0           | 3           | 1             | 1       | 2           |
| FLK  | AT3G04610        | 5           | 4           | 1             | 1       | 3           |
| GI   | AT1G22770        | 22          | 15          | 5             | 7       | 1           |
| LFY  | AT5G61850        | 4           | 4           | 1             | 1       | 1           |
| LHY  | AT1G01060        | 0           | 0           | 1             | 0       | 0           |
| VIN3 | AT5G57380        | 0           | 0           | 1             | 0       | 0           |
| SOC1 | AT2G45660        | 15          | 12          | 4             | 4       | 6           |
| TFL  | AT5G03840        | 24          | 13          | 6             | 5       | 7           |
| SVP  | AT1G24260        | 48          | 33          | 8             | 7       | 17          |
| PHYA | AT1G09570        | 10          | 5           | 3             | 3       | 4           |
| PHYB | AT2G18790        | 10          | 5           | 4             | 3       | 5           |
| PHYC | AT5G35840        | 0           | 1           | 1             | 1       | 4           |
| PHYE | AT4G18130        | 5           | 3           | 3             | 1       | 2           |

H. mutabilis was most closely related to H. syriacus, with an estimated divergence time of approximately 15.3 (10.1–21.5) Mya (Figure 5A). In addition to the paleohexaploidization event shared by the eudicots, we observed three additional whole-genome duplication (WGD) events in H. mutabilis. Hibiscus and Gossypium shared a WGD event (13.3–20.0 Mya), H. mutabilis and H. syriacus shared a WGD event (10.76–21.51 Mya), and H. mutabilis and H. syriacus each underwent a WGD event (4.61–9.00 Mya) (Figure 5B).

Flowering Time and Disease Resistance Genes

Genetic and molecular mechanisms of floral development are highly conserved among different plant species (Schiessl et al., 2014) and include four major flowering pathways that have been well characterized in A. thaliana. H. mutabilis is similar to H. syriacus, a short-day flowering plant with a long bloom period that produces more than 30 blossoms per day. Like those of H. syriacus, the flowers of H. mutabilis open daily and last for less than 48 h. Because flowering time is frequently dependent on gene copy number (Grover et al., 2015), we investigated the copy numbers of genes involved in the four major flowering pathways in A. thaliana, T. cacao, G. raimondii, A. trichopoda, H. syriacus, and H. mutabilis. Copy numbers of most flowering-related genes were higher in H. mutabilis than in other plants, including T. cacao, G. raimondii, A. trichopoda, and H. syriacus. In particular, the copy number of the plant-specific nuclear protein GIGANTEA (GI) was three to four times greater in H. mutabilis than in A. trichopoda, T. cacao, G. raimondii, and H. syriacus (Table 1).

Nucleotide-binding site (NBS) and carboxy-terminal LRR domains are found in the majority of R proteins (DeYoung and Innes, 2006; Takken et al., 2006). Based on resistance domain analyses in the H. mutabilis genome, a total of 490 NBS-containing resistance genes were identified and classified into six groups: CC-NBS-LRR, CC-NBS, TIR-NBS-LRR, TIR-NBS, NBS-LRR, and NBS. In total, their gene numbers were approximately three times greater in H. mutabilis than in A. thaliana (170). This trend was particularly striking for the NBS genes, whose numbers were much higher in H. mutabilis (192 genes) than in H. syriacus (54), T. cacao (9), G. raimondii (4), and A. thaliana (3). Although H. mutabilis had the highest number of NBS-containing resistance genes among the five angiosperms (Table 2), its number of NBS-containing genes as
FIGURE 6 | Heatmaps show the expression of key genes in the anthocyanin biosynthesis pathway of H. mutabilis. (A) Gene expression changes in flowers of H. mutabilis ‘Drunk girl’ as their color transitions from white to fully pink. (B) Gene expression in flowers of three different color cultivars: ‘single-petal white’ (SPW), ‘single-petal pink’ (SPP), and ‘Purple silk’ (PS).

Table 1: Heatmap showing gene expression changes in anthocyanin-related genes.

| Gene | Stage 1 | Stage 2 | Stage 3 |
|------|--------|--------|--------|
| HmCHS | 0.5    | 0.7    | 1.0    |
| HmCHI | -0.5   | 0.0    | 0.5    |
| HmF3H | 0.2    | 0.4    | 0.6    |
| HmF3’S| 0.8    | 1.0    | 1.2    |
| HmDFR | 0.1    | 0.3    | 0.5    |
| HmANS | -0.3   | 0.0    | 0.3    |
| Hm3GT | -0.7   | -0.5   | -1.0   |

A number of key anthocyanin biosynthesis-related genes, such as HmCHS, HmCHI, and HmANS, increased in expression from Stage 1 to Stage 3, consistent with the pattern of floral color development.

Key anthocyanin biosynthesis-related genes also differed in expression among different color cultivars of H. mutabilis, including ‘single-petal white’, ‘single-petal pink’, and ‘Purple silk.’ The highest expression levels were generally found in ‘Purple silk’, which is a deep purple color form (Figure 6B).

DISCUSSION

Completeness and continuity are important indicators of genome assembly quality. In this study, we took advantage of the longer read lengths offered by ONT sequencing that have proven advantageous in the assembly of other plant genomes such as Solanum pennelli (Schmidt et al., 2017) and Chrysanthemum nankingense (Song et al., 2018). Here, we report the first genome data for H. mutabilis and estimate its genome size to be 2.68 Gb, far larger than that of H. syriacus. Our H. mutabilis genome assembled using Nanopore reads had a contig N50 of 2.02 Mb. We then used Hi-C data to cluster the contigs into forty-six chromosomes with a final scaffold N50 of 54.70 Mb. The genome contained complete copies of 92.6% of the BUSCO orthologs examined. This genome sequence will contribute to our understanding of the biosynthesis of natural products such as anthocyanins, although additional research is needed to directly link specific genes to individual traits. Nonetheless, our high-quality, annotated genome sequence provides insights into determinate flowering time and flower color in H. mutabilis.

Compared with the H. syriacus genome, the H. mutabilis genome was larger and contained more protein-coding genes. H. mutabilis and H. syriacus share an MRCA approximately 15.3 (10.1–21.5) Mya, and investigation of WGD timing in the H. mutabilis genome showed that two WGDs occurred after H. mutabilis–H. syriacus divergence and H. mutabilis speciation. WGD events and tandem duplications are the most important determinants of genome size variation in angiosperms (Piegu et al., 2006; El Baidouri and Panaud, 2013). This recent WGD event not only caused genome expansion in H. mutabilis, but may also have contributed to the morphological and physiological diversity of H. mutabilis. We inferred that gene losses, which had different frequencies in H. mutabilis and H. syriacus, made the H. syriacus genome smaller than that of H. mutabilis. H. mutabilis has a long bloom period and high blossom turnover. The copy numbers of most flowering-related genes, such as GI, CONSTANS (CO), and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) were higher in H. mutabilis than in T. cacao, A. thaliana, and H. syriacus. These results show that...
"H. mutabilis" preserved many copies of flowering-related genes during the transition from a polyploid to a diploid genome.

The dynamic color change from white to pink in "H. mutabilis" ‘Drunk girl’ flowers is reported to be caused by variations in anthocyanin contents (Liu et al., 2008). Flavonoids are the major molecules involved in plant pigmentation (Lai et al., 2014) and include anthocyanins, flavan-3-ols (catechins and proanthocyanidins), flavanones, flavonols, flavones, and phenolic acid (Lou et al., 2014). To date, regulation of the flavonoid pathway has been shown to occur primarily at the transcriptional level (Mol et al., 1998). Different species have distinct regulated genes, and these appear to be among the most important candidate genes for flower color determination (Casimiro-Soriguer et al., 2016; Jiao et al., 2020). To investigate the expression of anthocyanin-related genes over the course of flower development and in different color forms, we combined high-quality genome sequence generated here with RNA-seq data from "H. mutabilis". The expression levels of anthocyanin biosynthetic genes such as HmcHs, HmChI, and HmAn were correlated and increased as flowers transitioned from white to pink. The pink flower color in cotton rose is related to the synthesis of cyanidin-based pigments (Chen et al., 2014), and our results indicate that low CHS, CHI, and ANS expression may inhibit cyanidin production in white flowers. Thus, combined genomic and transcriptomic analysis of "H. mutabilis" flowers indicated that structural genes had important roles in anthocyanin biosynthesis during the transition from white to pink flower coloration. In maize, an MYB-related protein and a bHLH containing protein interact to activate genes in the anthocyanin biosynthetic pathway (Schwinn et al., 2006). However, the functions of transcription factors, including MBYs, bHLHs, and WD40s, are unknown in "H. mutabilis". The investigation of these TFs in cotton rose will be a subject for further research.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/Genbank/, PRJNA717149.

AUTHOR CONTRIBUTIONS

YY and XLL: conceptualization. XG, MZ, and XDL: formal analysis. FL and ZZ: project administration. JM and XZ: resources. YY and XS: writing—original draft. XLL: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

SUPPLEMENTAL MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.818206/full#supplementary-material

REFERENCES

Aggarwal, G., and Ramaswamy, R. (2002). Ab initio gene identification: prokaryote annotation with GeneScan and GLIMMER. J. Biosci. 27, 7–14. doi: 10.1007/BF02703679

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., et al. (2000). Gene ontology: tool for the unification of biology. Nat. Genet. 25, 25–29. doi: 10.1038/75556

Bairoch, A., and Apweiler, R. (2000). The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. Nucleic Acids Res. 28, 45–48. doi: 10.1093/nar/28.1.45

Benson, G. (1999). Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res. 27, 573–580. doi: 10.1093/nar/27.2.573

Binney, E., and Clamp, M. (2004). GeneWise and GenomeWise. Genome Res. 14, 988–995. doi: 10.1101/gr.186504

Burton, J. N., Adey, A., Patwardhan, R. P., Qiu, R., Kitzman, J. O., and Shendure, J. (2013). Chromosome-scale scaffolding of de novo genome assemblies based on chromatin interactions. Nat. Biotechnol. 31, 1119–1125. doi: 10.1038/nbt.2772

Casimiro-Soriguer, I., Narbona, E., Buide, M. L., Del Valle, J. C., and Whittall, J. B. (2016). Transcriptome and biochemical analysis of a flower color mutant in "Hibiscus mutabilis". Nucleic Acids Res. 44, 7213–7227. doi: 10.1093/nar/gkw752

Chen, Y., Mao, Y., Liu, H., Yu, F., Li, S., and Yin, T. (2014). Transcriptome analysis of differentially expressed genes relevant to variegation in peach flowers. PLoS One 9:e90842. doi: 10.1371/journal.pone.0090842

De Bie, T., Cristianini, N., Demuth, J. P., and Hahn, M. W. (2006). CAFE: a computational tool for the study of gene family evolution. Bioinformatics 22, 1269–1271. doi: 10.1093/bioinformatics/btl097

DeYoung, B. J., and Innes, R. W. (2006). Plant NBS-LRR proteins in pathogen sensing and host defense. Nat. Immunol. 7, 1243–1249. doi: 10.1038/ni

Dubin, M., Lundy, K., Morrell, P., Torres-Martinez, C., and Clegg, M. (2004). Genes that determine flower color: the role of regulatory changes in the evolution of phenotypic adaptations. Mol. Phylogenet. Evol. 29, 507–518. doi: 10.1016/S1055-7903(03)00196-9

Edgar, R., and Myers, E. (2005). PILER: identification and classification of genomic repeats. Bioinformatics 21, 152–158. doi: 10.1093/bioinformatics/bti003

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797. doi: 10.1093/nar/gkh340

El Baidouri, M., and Panaud, O. (2013). Comparative genomic paleontology across plant kingdom reveals the dynamics of TE-Driven genome evolution. Genome Biol. Evol. 5, 954–965. doi: 10.1093/gbe/evt025

Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., et al. (2013). Pfam: the protein families database. Nucleic Acids Res. 42, D222–D230. doi: 10.1093/nar/gkt1223

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 29, 644–652. doi: 10.1038/nbt.1883

Griffiths-Jones, S., Moxon, S., Marshall, M., Khanna, A., Eddy, S. R., and Bateman, A. (2005). Pfam: annotating non-coding RNAs in complete genomes. Nucleic Acids Res. 33, 121–124. doi: 10.1093/nar/gki081

Grover, C. E., Gallagher, J. P., and Wendel, J. F. (2015). Candidate gene identification of flowering time genes in cotton. Plant Genome 8:plg00008. doi: 10.1002/plg2.20008

Haas, B. J., Delcher, A. L., Mount, S. M., Wortman, J. R., Smith, R. K. Jr., Hannick, L. I., et al. (2003). Improving the de novo assembly of whole genomes via repeat resolution. Genome Res. 13, 698–705. doi: 10.1101/gr.108601

Haas, B. J., Salzberg, S. L., Zhu, W., Pertea, M., Allen, J. E., Orvius, J., et al. (2008). Automated eukaryotic gene structure annotation using EvidenceModeler and the program to assemble spliced alignments. Genome Biol. 9:R7. doi: 10.1186/gb-2008-9-1-r7
Jiao, F., Zhao, L., Wu, X., Song, Z., and Li, Y. (2020). Metabolome and transcriptome analyses of the molecular mechanisms of flower color mutation in tobacco. BMC Genom. 21:611. doi: 10.1186/s12864-020-07028-5

Kanehisa, M., and Goto, S. (2000). KEGG: encyclopedia of genes and genomes. Nucleic Acids Res. 28, 27–30. doi: 10.1093/nar/28.1.27

Kim, Y. M., Kim, S., Koo, N., Shin, A. Y., Yoon, S. I., Seo, E., et al. (2016). Genome analysis of Hibiscus syriacus provides insights of polyploidization and indeterminate flowering in woody plants. DNA Res. 24, 71–80. doi: 10.1093/dnares/dsw049

Koes, R., Quattrocchio, F., and Mol, J. (1994). The flavonoid biosynthetic pathway in plants: Function and evolution. Bioessays 16, 123–132. doi: 10.1002/bies.950160209

Korf, I. (2004). Gene finding in novel genomes. BMC Bioinf. 5:59. doi: 10.1186/1471-2105-5-59

Lai, B., Li, X. J., Hu, B., Qin, Y. H., Huang, X. M., Wang, H. C., et al. (2014). LoMYBI is a key determinant of differential anthocyanin accumulation among genotypes, tissues, developmental phases and ABA and light stimuli in Litchi chinensis. PLoS One 9:e86293. doi: 10.1371/journal.pone.0086293

Li, H. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760. doi: 10.1093/bioinformatics/btp324

Li, L., Stoeckert, C. J. Jr., and Roos, D. S. (2003). OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. 13, 2178–2189. doi: 10.1101/gr.122450

Li, X., Tang, D., Du, H., and Shi, Y. (2018). Transcriptome sequencing and biochemical analysis of perianth and corona reveal flower color formation in Narcissus pseudonarcissus. Int. J. Mol. Sci. 19:4006. doi: 10.3390/ijms19124006

Li, Y. P., Zhang, X. L., Wu, W. T., Miao, S. X., and Chang, J. L. (2015). Chromosome formation in chrysanthemum petals. J. Exp. Bot. 65, 3157–3164. doi: 10.1093/jxb/eru168

Lowe, T. M., and Eddy, S. R. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 25, 955–964. doi: 10.1093/nar/25.5.955

Lupas, A., Van Dyke, M., and Stock, J. (1991). Predicting coiled coils from protein sequences. Science 252, 1162–1164. doi: 10.1126/science.252.5009.1162

Majors, W., Perta, M., and Salzberg, S. (2004). TigrScan and GlimmerHMM: two open source ab initio eukaryotic gene-finders. Bioinformatics 20, 2878–2879. doi: 10.1093/bioinformatics/bth315

Marchin, M., Kelly, P. T., and Fang, J. (2005). Tracker: continuous HMMER detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 33, W465–W467. doi: 10.1093/nar/gki458

Takken, F. L., Albrecht, M., and Tameling, W. I. (2006). Resistance proteins: molecular switches of plant defence. Curr. Opin. Plant Biol. 9, 383–390. doi: 10.1016/j.pbi.2006.05.009

Tang, H., Bowers, J. E., Wang, X., Ming, R., Alam, M., and Paterson, A. H. (2008). Synteny and collinearity in plant genomes. Science 320, 486–488. doi: 10.1126/science.1153917

Tarailo-Graovac, M., and Chen, N. (2009). Using RepeatMasker to identify repetitive elements in genomic sequences. Bioinformatics 25, 1105–1111. doi: 10.1093/bioinformatics/btp312

Trapnell, C., Pachter, L., and Salzberg, S. (2009). TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25, 1105–1111. doi: 10.1093/bioinformatics/btp120

Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M. J., et al. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 28, 511–515. doi: 10.1038/nbt.1621

Vaser, R., Sofic, I., Nagarajan, N., and Sikic, M. (2017). Fast and accurate de novo genome assembly from long uncorrected reads. Genome Res. 27, 737–746. doi: 10.1101/gr.214270.116

Walker, B., Abeel, T., Shea, T., Priest, M., Aboueliel, A., Sakthikumar, S., et al. (2014). Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PLoS One 9:e112963. doi: 10.1371/journal.pone.0112963

Wang, K., Wang, Z., Li, F., Ye, W., Wang, J., Song, G., et al. (2012). The draft genome of a diploid cotton Gossypium raimondii. Nat. Genet. 44, 1098–1103. doi: 10.1038/ng.2371

Wang, M., Tu, L., Yuan, D., Zhu, D., Shen, C., Li, J., et al. (2019). Reference genome sequences of two cultivated allotetraploid cottons, Gossypium hirsutum
and Gossypium barbadense. Nat. Genet. 51, 224–229. doi: 10.1038/s41588-018-0282-x

Wikström, N., Savolainen, V., and Chase, M. W. (2001). Evolution of the angiosperms: calibrating the family tree. Proc. Biol. Sci. 268, 2211–2220. doi: 10.1098/rspb.2001.1782

Xu, Z., and Wang, H. (2007). LTR-FINDER: an efficient tool for the prediction of full-length LTR retrotransposons. Nucleic Acids Res. 35, W265–W268. doi: 10.1093/nar/gkm286

Yang, T. J., Kim, J. S., Lim, K. B., Kwon, S. J., Kim, J. A., Jin, M., et al. (2005). The Korea Brassica genome project: a glimpse of the Brassica genome based on comparative genome analysis with Arabidopsis. Comp. Funct. Genom. 6, 138–146. doi: 10.1002/cfg.465

Yang, Z. (1997). PAML: a program package for phylogenetic analysis by maximum likelihood. Bioinformatics 13, 555–556. doi: 10.1093/bioinformatics/13.5.555

Yu, X. J., Zheng, H. K., Wang, J., Wang, W., and Su, B. (2007). Detecting lineage-specific adaptive evolution of brain-expressed genes in human using rhesus macaque as outgroup. Genomics 88, 745–751. doi: 10.1016/j.ygeno.2006.05.008

Zhang, B., Liu, C., Wang, Y., Yao, X., Wang, F., Wu, J., et al. (2015). Disruption of a CAROTENOID CLEAVAGE DIOXYGENASE 4 gene converts flower colour from white to yellow in Brassica species. New Phytol. 206, 1513–1526. doi: 10.1111/nph.13335

Zhang, L., Xu, Y., Zhang, X., Ma, X., Zhang, L., Liao, Z., et al. (2020). The genome of kenaf (Hibiscus cannabinus L.) provides insights into bast fiber and leaf shape biogenesis. Plant Biotechnol. J. 18, 1796–1809. doi: 10.1111/pbi.13341

Zhao, M., and Ma, J. (2013). Co-evolution of plant LTR-retrotransposons and their host genomes. Prot. Cell 4, 493–501. doi: 10.1007/s13238-013-3037-6

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