

Navigating the Mulberry Genome: Integrating QTL Mapping and RNA-seq for Trait Discovery in *Morus indica*

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ABSTRACT

Gene annotation, QTL mapping, and gene expression analysis play crucial roles in understanding the genetic basis of complex traits in mulberry (*Morus indica*) varieties. Through the integration of these approaches, researchers gain insights into gene functions, biological processes, and pathway associations, facilitating targeted crop improvement strategies. The lack of molecular markers has historically impeded selective breeding efforts in mulberry; however, the utilization of SSRs, SNPs, and genetic linkage mapping has provided valuable insights into genetic diversity and population structure. By leveraging molecular tools, researchers aim to enhance mulberry's contribution to agriculture and industry while ensuring its sustainable development. The presented genomic information, including gene ontology, pathway associations, and expression values, highlights the genetic basis of key traits in mulberry varieties. Furthermore, the importance of QTL mapping and gene expression analysis in identifying genomic regions associated with traits such as yield, disease resistance, and stress

tolerance is emphasized. These techniques serve as essential tools in modern agriculture for enhancing crop productivity, sustainability, and resilience to environmental stresses.

Key words: *Gene annotation, QTL Mapping, Gene expression, DGE, Markers*

1. Introduction:

The mulberry (*Morus*), a resilient perennial woody plant within the Moraceae family, has garnered attention for its rapid growth and diverse species. Among these, *Morus indica*, a deciduous tree native to the Himalayan region and widely cultivated in India, China, Japan, and East Africa, stands out for its significant role in sericulture [1]. Characterized by its prolific leaf production and adaptability to various environments, *Morus indica* plays a vital role in providing nourishment for silkworms, thereby influencing silk production [2]. Beyond its economic significance, *Morus* species contribute to ecological sustainability through their disease-resistant compounds [3]. The intricate interplay between tradition, innovation, and ecological balance in sericulture underscores the importance of understanding and enhancing *Morus* genetic traits. With over 24 species and 1000 cultivars worldwide, *Morus* exhibits extensive genetic diversity, which has been harnessed for centuries through selective breeding programs aimed at improving leaf yield, disease resistance, and adaptability [5]. However, traditional breeding methods are often time-consuming and imprecise, prompting the exploration of modern genetic techniques for mulberry improvement [6]. Quantitative Trait Loci (QTL) analysis has emerged as a powerful tool in plant genetics, particularly for studying complex traits like those found in mulberry [6]. By detecting associations between phenotypic traits and genetic markers, QTL analysis allows researchers to identify genomic regions influencing quantitative traits such as leaf yield, silk production, and disease resistance [7]. Linkage analysis and association mapping, two commonly used methods for QTL mapping, help identify subsets of markers associated with specific QTLs regulating complex trait expression [6]. Techniques like Single Nucleotide Polymorphism (SNP) analysis facilitate the identification of genetic variations associated with traits such as drought tolerance in mulberry [8]. In parallel, RNA sequencing (RNA-seq) has revolutionized our ability to understand gene expression patterns and molecular mechanisms underlying complex traits in plants like *Morus indica* [11, 8]. By sequencing the entire transcriptome, RNA-seq enables the simultaneous measurement of thousands of gene expression levels, offering insights into crucial agronomic traits such as leaf yield, silk production, and disease resistance [11]. Integration of QTL

analysis and RNA-seq represents a promising approach for elucidating the genetic basis of complex traits in *Morus indica* [14]. QTL analysis identifies genomic regions associated with traits, while RNA-seq provides insights into the functional genes and pathways within those regions [15]. This integrative approach has been successful in other plant species, leading to the identification of candidate genes for various traits [14, 16]. In the context of *Morus indica* genetic improvement, the integration of QTL analysis and RNA-seq holds immense potential for accelerating targeted breeding efforts and ensuring the sustainability of the sericulture industry. High-yielding, disease-resistant varieties not only enhance silk production but also promote ecological sustainability by reducing the need for chemical pesticides [17, 18]. Focused research efforts on key phenotypes like leaf yield, nitrogen utilization efficiency, drought tolerance, and root rot resistance, coupled with gene prediction studies and gene ontology analyses, pave the way for a comprehensive understanding of *Morus* genetic traits. Integration of RNA-seq data analysis with gene ontology results facilitates the study of Expression Quantitative Trait Loci (E-QTL), shedding light on the genetic effects underlying these phenotypes. In conclusion, the integration of QTL analysis and RNA-seq in *Morus indica* genetic improvement holds promise for sustainable agriculture and the prosperity of sericulture-dependent communities. By unravelling the genetic mechanisms governing complex traits, we can pave the way for the development of resilient, high-yielding mulberry varieties, ensuring the long-term viability of silk production and the well-being of rural economies.

2. Methodology

2.1 Sample Collection

The RNA samples utilized in this study were sourced from leaf tissues of distinct mulberry (*Morus indica*) varieties, including k2, Thailand male, S1, and assama bola. These samples were extracted from the *Morus indica* Genome Project (MindGP) database, a comprehensive repository of genomic and transcriptomic data for mulberry species [19]. The selection of mulberry varieties for RNAseq analysis aimed to capture genetic diversity and phenotypic variation relevant to sericulture and agricultural practices. Each variety was chosen based on its genetic background and potential significance in mulberry breeding programs [20]. The sampling strategy prioritized including representative samples from diverse geographical regions to encompass the broad genetic landscape of mulberry cultivation. Leaf tissues representing each mulberry variety were obtained from the MindGP database, ensuring access

to a wide range of genotypic and phenotypic data. Accession codes corresponding to the genome assemblies of the selected varieties were used to retrieve the respective sequences. Metadata accompanying the genome sequences, such as assembly statistics and annotations, were obtained for downstream analysis.

2.2 Gene Prediction

Gene prediction, also known as gene finding or gene annotation, is the process of identifying the locations and structures of protein-coding genes within a genome. In other words, it involves computationally identifying regions of DNA sequence that encode proteins or functional RNA molecules. Gene prediction analysis was performed using the AUGUSTUS tool. The genome sequences of k2, Thailand male, S1, and assama bola were input into AUGUSTUS for ab initio gene prediction. AUGUSTUS employs hidden Markov models trained on species-specific gene structures to predict protein-coding genes within genomic sequences [21]. Default parameters were utilized, and predicted gene models were generated for each *Morus indica* variety. The predicted gene models generated by AUGUSTUS were annotated using bioinformatics databases and tools.

2.3 RNA-seq Analysis

RNA-seq DGE (Differential Gene Expression) analysis, also known as RNA-seq DE (Differential Expression) analysis, is a method used to identify genes that are differentially expressed between different experimental conditions or biological states based on RNA-seq data [22]. RNA-seq analysis was conducted to characterize gene expression patterns associated with key phenotypes such as leaf yield, nitrogen utilization efficiency, drought tolerance, and root rot resistance. The RNA-seq reads obtained from the k2, Thailand male, S1, and assama bola varieties were aligned to the *Morus indica* reference genome using HISAT2, followed by counting reads with FeatureCounts and differential gene expression (DGE) analysis using edgeR [23]. Alignment files were generated for each sample, facilitating the quantification of gene expression levels. FeatureCounts assigned reads to genes based on *Morus indica* gene annotations obtained from the MindGP database, and the resulting count table enabled the identification of differentially expressed genes between varieties. This integrated approach allows for the identification of genes associated with important agronomic traits.

2.4 Gene Ontology

Gene Ontology (GO) analysis is an essential component of our study, facilitating a systematic exploration of gene function and biological processes associated with key phenotypic traits in *Morus indica* varieties. QuickGO, a user-friendly online tool provided by the Gene Ontology Consortium, is utilized for this purpose [24]. With QuickGO, genes from *Morus indica* varieties (such as k2, Thailand male, S1, and assama bola) are annotated with standardized GO terms representing their molecular functions, biological processes, and cellular components. This annotation process leverages curated GO annotations available in the QuickGO database, ensuring comprehensive coverage of gene functions relevant to our study. QuickGO enables the identification of enriched GO terms among differentially expressed genes identified through RNA-Seq analysis. By inputting lists of differentially expressed genes into QuickGO, functional enrichment analysis is performed to uncover GO terms that are significantly overrepresented within the gene set. This analysis provides insights into the biological processes and molecular functions associated with the studied phenotypic traits. The enriched GO terms obtained from QuickGO analysis offer valuable insights into the biological mechanisms underlying trait manifestation in *Morus indica* varieties. By examining the enriched GO terms, we can elucidate the functional roles of differentially expressed genes and discern the regulatory networks governing the expression of key phenotypic traits, such as leaf yield, nitrogen utilization efficiency, drought tolerance, and root rot resistance. Integration of RNA-seq data analysis with QuickGO results allows us to correlate gene expression levels with phenotypic variation observed in *Morus indica* varieties. By linking differentially expressed genes to specific GO terms representing relevant biological processes, we aim to unravel the regulatory networks and pathways that modulate the expression of target traits [25].

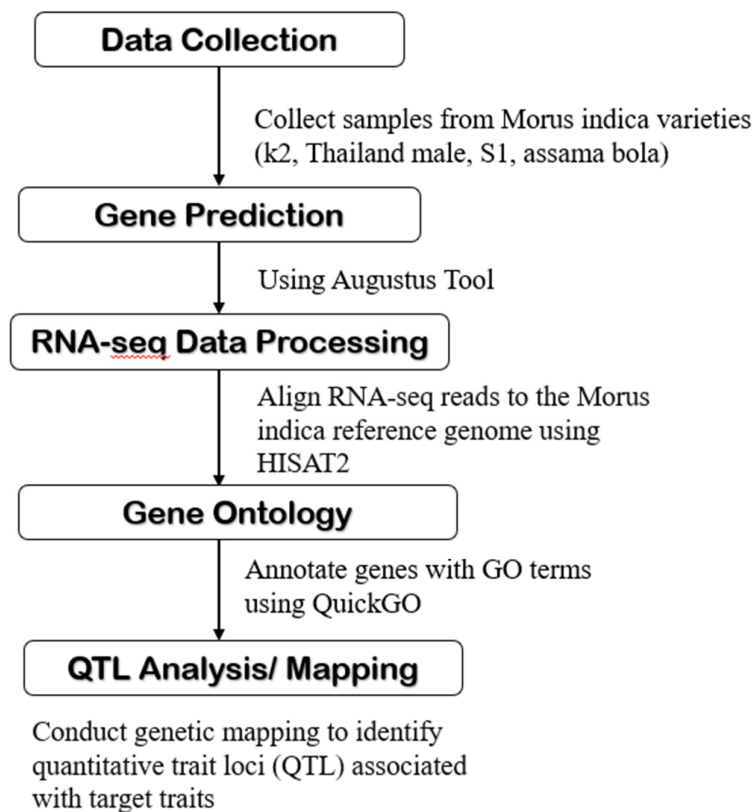
2.5 Mapping

The study focuses on the QTL analysis of *Morus indica* varieties, a crucial step involves the mapping of RNA-seq reads obtained from k2, Thailand male, S1, and assama bola varieties to the *Morus indica* reference genome. This process enables the quantification of gene expression levels and subsequent analysis of differential gene expression associated with key phenotypic traits [26]. RNA-seq reads from each *Morus indica* variety are aligned to the *Morus indica* reference genome using the HISAT2 alignment tool [27]. HISAT2 employs a fast and sensitive algorithm to accurately map short RNA-seq reads to the reference genome, considering potential splice junctions and genomic features specific to *Morus indica*.

During alignment, HISAT2 is configured to optimize alignment parameters, such as seed length, seed spacing, and maximum number of mismatches, to achieve robust and accurate mapping of RNA-seq reads. Additionally, parameters related to the reference genome index are specified to facilitate efficient alignment. Following alignment, quality control checks are performed to assess the quality of the alignment results. Metrics such as mapping rates, alignment scores, and distribution of mapped reads across genomic features (e.g., exons, introns) are evaluated to ensure the reliability of the mapping process. Aligned reads are stored in BAM (Binary Alignment/Map) format, which provides a compressed and indexed representation of the alignment data. BAM files contain information about the genomic coordinates where each read aligns, as well as alignment quality scores and other relevant metadata. Visualization facilitates the inspection of alignment patterns, identification of potential alignment artifacts, and validation of mapping results.

2.6 QTL mapping

From the previous studies based on the SNPs and linkage mapping studies, the genes which were correlating with the phenotypes: Drought, Leaf yield, Nitrogen use efficiency and Root rot were chosen and their expression was seen

Figure 1 Methodology implemented for QTL analysis

3. RESULTS

3.1 Selection of mulberry genomes

The *Morus indica* genomes were collected from ENA database based on the phenotype relations.

Table 1 Genetic Varieties and Their Traits in Response to Environmental Stresses

Genotype name	Drought (NW2b)	Leaf Yield	NUE (NW2d)	Root rot (NW2e)
K2	Medium	Low	-	Moderately Resistant
S1	Not included	Medium	High	Highly susceptible

Thailand Male	Not included	Medium	Moderate	Highly susceptible
Assama Bola	Medium	High	Low	Moderately susceptible

In agricultural landscapes, the quest for resilient crop varieties is paramount, particularly in the face of escalating environmental challenges such as drought, nutrient use efficiency (NUE), and root rot. The following genotype table showcases key traits of various plant varieties concerning these stressors:

The K2 genotype displays a moderate tolerance to drought stress, suggesting its potential to withstand periods of water scarcity. However, its low leaf yield may indicate a compromise in overall productivity. Notably, its resistance to root rot is a positive attribute, enhancing its suitability for cultivation in environments prone to soil-borne pathogens.

S1 exhibits a commendable balance between leaf yield and NUE, indicating efficient resource utilization. However, the absence of data regarding its drought tolerance raises concerns about its adaptability to water-limited conditions. Moreover, its high susceptibility to root rot may necessitate careful management practices to mitigate disease impact.

Similar to S1, Thailand Male demonstrates promising leaf yield and moderate NUE. Nonetheless, its susceptibility to root rot poses a significant risk, potentially compromising plant health and productivity in pathogen-infested soils.

Assama Bola emerges as a robust genotype with a high leaf yield, indicating its capacity for substantial biomass production. However, its lower NUE suggests a potential inefficiency in nutrient utilization. While moderately susceptible to root rot, its medium drought tolerance underscores its adaptability to water-stressed environments.

In summary, the presented genotypic traits underscore the complex interplay between various stressors and plant performance. Efforts aimed at breeding and selecting cultivars with superior traits, including drought tolerance, high yield potential, and disease resistance, remain crucial for ensuring agricultural sustainability and food security in the face of evolving environmental pressures.

3.2 Gene Annotation

The genomes were annotated to know the functionality of the genes. A total of 75,000 genes were identified in the UTR, genomic and exon regions. The complete gene annotation is shown below:

[Genome annotation](#)

3.3 Gene Ontologies

The complete biological process, molecular functions and the cellular components were identified. The complete gene ontology studies is shown below:

[Gene ontology Morus indica](#)

3.4 Differential gene expression and QTL Mapping

The complete differential gene expression is shown below:

[Differential gene expression](#)

The mapping results are shown below:

[Linkage mapping.xlsx](#)

4. DISCUSSION

Gene annotation is pivotal in QTL studies, aiding in candidate gene identification, functional inference, and pathway elucidation. Annotated genes streamline marker development, enhance comparative genomic analyses, and guide functional validation experiments. Moreover, they bolster genomic selection models, refining prediction accuracy for breeding programs. Through this multi-faceted approach, gene annotation expedites the unravelling of complex trait genetics, facilitating targeted crop improvement strategies.

Gene ontologies are indispensable tools in QTL mapping studies, offering structured vocabularies for gene function, biological processes, and cellular components. They facilitate functional annotation by categorizing genes, aiding in the identification of candidate genes within QTL regions and streamlining subsequent validation efforts. Through gene ontologies, researchers gain insights into the biological mechanisms underlying trait variation, guiding hypothesis generation and experimental design. Additionally, ontologies aid in prioritizing candidate genes, directing attention to those involved in relevant biological pathways. Pathway analysis benefits from ontological organization, allowing for a comprehensive understanding

of the genetic architecture of target traits. Comparative genomics is enhanced by standardized ontological frameworks, facilitating cross-species comparisons and elucidating conserved biological processes. Gene ontologies also guide functional validation experiments by assisting in the selection of genes for experimental validation, thus improving the efficiency of validation studies. Overall, gene ontologies play a vital role in QTL mapping, contributing to functional annotation, biological insights, candidate gene prioritization, pathway analysis, comparative genomics, and functional validation, thereby accelerating crop improvement efforts. *Morus*, commonly known as mulberry, holds immense significance for crop enhancement and sustainable development. Despite its genetic diversity, the lack of molecular markers has impeded selective breeding efforts. To overcome this, SSRs and SNPs are employed to study genetic diversity and population structure. Genetic linkage mapping in *Morus indica* is pivotal, aiming to identify relevant markers and develop specialized microchips. This research offers insights into genetic diversity and adaptations, informing crop improvement strategies. By leveraging molecular tools, we can enhance mulberry's contribution to agriculture and industry, ensuring its sustainable development and broader applications. The provided table presents genomic information related to different genes, their ontology, pathway associations, genetic variations (reference and alternate alleles), associated breeds, and phenotypic expressions. Each entry signifies a specific genomic locus, highlighting its relevance to certain traits or biological processes in particular mulberry varieties. For instance, the gene Mi00022 is associated with the ontology "Response to Water Deprivation," indicating its involvement in drought response mechanisms. This gene is identified in Assama Bola, suggesting its potential role in conferring drought tolerance in this variety. Similarly, the gene Mi00043 is linked to "Nitrogen Fixation," essential for nitrogen utilization efficiency (NUE), particularly relevant in Assama Bola. This highlights the genetic basis of NUE traits in mulberry. The table also reveals associations between genes and traits like leaf yield and root rot resistance. For example, genes Mi00041 and Mi00012 are associated with "Chlorophyll Binding" and "Carbon Fixation," respectively, potentially influencing leaf yield in varieties S1 and Thailand male.

Additionally, These findings underscore the significance of understanding the genetic basis of key traits in mulberry varieties. Utilizing genomic information can aid in targeted breeding programs, enhancing traits like drought tolerance, NUE, and disease resistance, thereby contributing to the sustainable development of the mulberry industry. Furthermore, such insights can inform molecular breeding strategies aimed at improving mulberry varieties for

diverse agricultural and industrial applications. Expression values indicate the level of gene activity or transcript abundance associated with specific traits or conditions. These values quantify the extent to which genes are being expressed or activated in response to environmental factors or developmental stages. By analysing expression values across different genotypes or phenotypic conditions, researchers can identify candidate genes that may contribute to trait variation. Expression values serve as valuable indicators of gene function and regulation, helping elucidate the genetic mechanisms underlying complex traits and informing breeding strategies for trait improvement in agricultural crops like mulberry.

But the results obtained from table 2 which exclusively was done on finding the only the expressions for K2 mulberry variety, the presence of necrosis-related gene Mi00007 in K2 indicates its susceptibility to root rot, a trait of concern in mulberry cultivation. While we are trying to find the Genotype phenotype relationship with the SNP markers we could find only relation with 3 phenotypes but when we looked into expressions and mapped it to the phenotypes all the 4 phenotypes showed some relation which shows the importance of QTL mapping. QTL mapping and gene expression analysis play pivotal roles in agricultural research and crop improvement strategies. QTL mapping helps identify genomic regions associated with important traits such as yield, disease resistance, and stress tolerance. Understanding these genetic regions allows for targeted breeding efforts to develop crop varieties with desired traits, contributing to enhanced productivity and sustainability.

Gene expression analysis provides insights into how genes are regulated and activated in response to various environmental cues and stresses. This knowledge is crucial for understanding the molecular mechanisms underlying trait expression and adaptation in crops. By studying gene expression patterns, researchers can identify key genes involved in important agronomic traits and develop molecular markers for marker-assisted selection (MAS) in breeding programs. Together, QTL mapping and gene expression analysis enable researchers to unravel the genetic basis of complex traits, accelerate breeding efforts, and develop resilient crop varieties capable of meeting the challenges of changing environmental conditions and increasing global food demand. These techniques are essential tools in modern agriculture for enhancing crop productivity, sustainability, and resilience to biotic and abiotic stresses.

5. CONCLUSION

In conclusion, the integration of gene annotation, gene ontologies, QTL mapping, and gene expression analysis provides a comprehensive framework for understanding the genetic basis

of complex traits in mulberry varieties. Through the elucidation of gene functions, biological processes, and pathway associations, researchers gain valuable insights into the mechanisms underlying trait variation. This knowledge not only informs targeted breeding programs for enhancing traits such as drought tolerance, nitrogen utilization efficiency, and disease resistance but also contributes to the sustainable development of the mulberry industry.

The findings underscore the importance of leveraging genomic information and molecular tools to drive crop improvement strategies. By identifying genomic regions associated with key traits and elucidating gene expression patterns, researchers can accelerate breeding efforts and develop resilient crop varieties capable of meeting the challenges of changing environmental conditions and increasing global food demand.

In summary, QTL mapping and gene expression analysis serve as indispensable tools in modern agriculture, enabling researchers to unravel the genetic basis of complex traits, enhance crop productivity, and ensure the sustainability of mulberry cultivation and its broader applications in agriculture and industry.

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8. CONFLICT OF INTEREST

The authors declare there is no conflict of interest

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