MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

QUALIFICATION THESIS

on the topic **Cloning and analysis of CoA ligase gene of hop *Humulus lupulus***
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group
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Ph.D., As. prof.

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Kyiv 2025

KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN

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APPROVE
Head of Biotechnology, Leather and Fur Department, Professor,
Dr. Sc., Prof.
_____Olena MOKROUSOVA
«___»_____2025

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Chen Xiaoxuan

1. Thesis topic **Cloning and analysis of CoA ligase gene of hop *Humulus lupulus***
Scientific supervisor Ph.D., Assoc. Prof. Iryna Voloshyna

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2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of Pre-graduation practice

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

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WORK CALENDAR

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	until 11 April 2025	
2	Chapter 1. Literature review	until 20 April 2025	
3	Chapter 2. Object, purpose, and methods of the study	until 30 April 2025	
4	Chapter 3. Experimental part	until 11 May 2025	
5	Conclusions	until 15 May 2025	
6	Draw up a bachelor's thesis (final version)	until 25 May 2025	
7	Submission of qualification work to the supervisor for feedback	until 27 May 2025	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	28 May 2025	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	01 June 2025	Similarity coefficient ____% Citation rate ____%
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	04 June 2025	

I am familiar with the task:

Student _____ Chen XIAOXUAN

Scientific supervisor _____ Iryna VOLOSHYNA

SUMMARY

Chen Xiaoxuan. Cloning and analysis of CoA ligase gene of hop *Humulus lupulus* – Manuscript.

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Hops are a key raw material in beer brewing, directly determining the flavor and quality of beer. This study took *Humulus lupulus 'Qingdao Dahua'* as the research object to carry out the cloning of flavor-related genes and bioinformatics analysis. Among them, isohumulone formed by the isomerization of *α-acid* is the main source of beer bitterness; *β-acid* helps improve the foam stability of beer; polyphenolic compounds affect the color, flavor stability and antioxidant properties of beer. In the experiment, healthy leaves of Qingdao Dahua hop plants were used as materials. After extracting total RNA by the TRIzol method and synthesizing the first strand of cDNA by reverse transcription, nested PCR technology was used to clone the target genes. Sequencing and alignment showed that the cloned genes had no differences from the sequences of the sequenced varieties, providing a reliable foundation for follow-up in-depth research. Through bioinformatics analysis, the physicochemical properties, secondary and tertiary structures of the proteins encoded by the target genes were predicted, and their functional domains and active sites were mined. The research results will provide theoretical support for revealing the flavor formation mechanism of hops, facilitate the genetic improvement of hop varieties, promote the optimization and upgrading of beer brewing processes, and be of great significance for the sustainable development of the beer industry.

Key words: Hops; Flavor substances; Gene cloning; Bioinformatics analysis; Beer brewing

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INTRODUCTION

Relevance of the Topic Hops, as the core raw material in beer brewing, have long been a research focus in the fields of food science and plant molecular biology regarding the synthesis mechanisms of their flavor compounds. The α-acids, β-acids, and polyphenolic compounds in hops not only impart beer with its distinctive bitterness, foam stability, and antioxidant properties but also directly influence beer quality and market competitiveness. In recent years, with increasing consumer demand for diversified beer flavors, elucidating the molecular mechanisms underlying the synthesis of hop flavor compounds has become crucial for optimizing beer brewing processes and driving industrial advancement.

Coenzyme A ligase, a key enzyme in the secondary metabolic network of hops, directly participates in the biosynthetic pathways of α-acids, β-acids, and polyphenolic compounds by catalyzing the linkage of substrates with coenzyme A. The expression level and functional activity of this gene play a decisive role in the accumulation of hop flavor compounds. However, current understanding of the cloning of hop coenzyme A ligase genes and their regulatory mechanisms in flavor formation remains incomplete, hindering progress in hop variety improvement through gene-editing technologies. Therefore, conducting cloning and bioinformatics analysis of hop coenzyme A ligase genes holds significant theoretical and practical importance for deciphering the molecular basis of hop flavor formation.

The purpose of the study This study aims to focus on Qingdao Magnum hops, with the objectives of cloning its CoA ligase gene and analyzing the sequence characteristics, protein structure, and evolutionary relationships of this gene, thereby revealing its function in the synthesis of hop flavor compounds. The findings will provide a theoretical basis for molecular breeding and flavor regulation in hops. Specific aspects include: establishing an efficient cloning technology system for the CoA ligase gene in hops, analyzing the sequence

conservation of this gene and the physicochemical properties as well as the higher-order structure of its encoded protein, and exploring the potential mechanistic role of this gene in the biosynthetic pathway of hop flavor compounds.

The objectives of the study

Gene Cloning and Sequence Analysis: Utilize nested PCR technology to clone the CoA ligase gene from the leaves of Qingdao Dahua hops, verify its sequence accuracy through sequencing and BLAST alignment, and analyze the gene's open reading frame and conserved domains.

Bioinformatics Prediction: Employ tools such as ProtScale, TMHMM, and SwissModel to predict the protein's hydrophobicity, transmembrane structure, secondary and tertiary structures, and elucidate its functional domains and catalytic mechanisms.

Functional Correlation Analysis: Integrate the NCBI database and String-DB tool to explore the interaction network between this gene and enzymes related to hop flavor compound synthesis, revealing its synergistic role in metabolic pathways.

The object of the study This study focuses on Humulus lupulus L. cv. Qingdao Dahua (Qingdao Dahua hops) as the research subject. This cultivar is a dual-purpose hops variety independently developed in China, suitable for both bittering and aroma applications. It is characterized by balanced α-acid and β-acid content (α/β ratio of 1.0-1.5), high total polyphenol content (approximately 7.0%), and abundant hop oil components such as humulene and caryophyllene. Its flavor profile combines floral, spicy, and herbal notes, making it suitable for brewing various beer styles. The experimental materials consisted of healthy leaves collected during the vigorous growth period, which were flash-frozen in liquid nitrogen and stored for RNA extraction and gene cloning.

Practical Significance of the Research This study focuses on the cloning and bioinformatics analysis of the CoA ligase gene in Qingdao Magnum hops,

yielding results of significant value in both theoretical exploration and practical applications.

At the theoretical level, the research systematically elucidates the sequence characteristics and protein structure of this gene for the first time, revealing 100% identity with homologous sequences in the NCBI database. The encoded protein contains an AMP-binding domain and a DXDD conserved motif, confirming its core catalytic role in the synthesis of α-acids, β-acids, and polyphenolic compounds. These findings refine the molecular regulatory network underlying hop flavor formation, provide a new model for studying secondary metabolic regulation mechanisms in perennial herbaceous plants, and enrich functional research on the plant CoA ligase gene family.

On the practical front, the study identifies a target gene for molecular breeding in hops. Utilizing CRISPR/Cas9 and other technologies, targeted regulation of gene expression can be achieved to cultivate varieties with high α-acid and terpene content, shortening traditional breeding cycles and reducing costs. Additionally, by analyzing the correlation between gene expression patterns and environmental factors, hop cultivation and brewing process parameters (e.g., harvest timing, boiling temperature) can be optimized to enhance flavor compound synthesis efficiency and beer quality. Furthermore, the research outcomes facilitate the development of proprietary hop varieties, advancing China's beer industry from empirical brewing to molecular design-based upgrades and strengthening international competitiveness.

From academic and societal perspectives, the study integrates multidisciplinary approaches from molecular biology, bioinformatics, and food science, serving as a model for interdisciplinary research. The precision improvement model based on gene editing reduces resource consumption in traditional breeding, supporting sustainable agricultural development. The research holds profound implications for theoretical innovation, technological advancement, and green transformation in the beer industry.

Chapter 1
LITERATURE REVIEW

1.1 Overview of hops

Hops are a key ingredient in beer brewing and are perennial climbing herbs of the genus Humulus, family Moraceae. It contains a variety of components that are crucial to the flavor of beer, like α-acids and β-acids that give beer its bitterness, with isomerized α-acids being the main source of bitterness; and polyphenols, which affect the color and flavor stability of beer with antioxidant properties. In addition, it contains terpenoids that bring floral, fruity and other aromas [*]. Hops not only shape the unique flavor of beer, but also have the role of stabilizing foam, inhibiting the growth of stray bacteria, etc., which has a great impact on the quality of beer.

People's knowledge and utilization of hops has gone through a long process. The cultivation of hops can be traced back to ancient Babylon around 200 BC, but it was not until 1079 AD that the Germans first added hops to beer brewing, which gave beer a refreshingly bitter flavor and an aftertaste[4]. Initially, beer was brewed without hops, and most beers of the time were sweet or cloying. In order to improve palatability and balance the flavor, people tried to add various ingredients with sour or bitter taste, and hops were one of them. In the competition with various fruits, spices and herbs, hops gradually emerged as a unique advantage. 11th century, hops have been used in beer brewing, but at that time the grout composed of aromatic prunes, yarrow and so on, dominated in balancing the flavor of beer [*]. Later, the Holy Roman Emperor Henry IV franchised glute, which led to higher beer costs, making the traditional glute recipe gradually abandoned, and hops took its place.In 1516, the German state of Bavaria enacted the Beer Purity Act, which is regarded as the cornerstone of the modern brewing regulations, promoting the standardization of hops cultivation and brewing process innovation, which stipulates that beer can only be used in the

use of water, yeast, malt and hops, which further established the importance of hops in beer brewing [*7].

1.2 Influence of hops on beer flavor

Hops occupy an irreplaceable central position in the beer brewing industry, and are the key raw material to give beer a unique flavor. The plant is rich in a variety of substances that have a significant impact on the flavor of beer, mainly including bitter substances, acids and polyphenolic compounds, etc., which interact and synergize with each other during the brewing process, and together shape the rich and complex taste and aroma of beer.

The bitterness in hops mainly comes from two important substances, alpha- and beta-acids. During the boiling process, which is a key part of beer brewing, α-acids undergo isomerization and are converted into iso-α-acids. Iso-α-acids are the main flavor presenting substances of beer bitterness, and their content in beer directly determines the intensity of beer bitterness [*]. Through precise control of brewing parameters such as the amount of hops, boiling time and temperature, the degree of isomerization of α-acids can be effectively regulated to precisely control the intensity of beer bitterness and satisfy different consumer preferences for beer bitterness. β-acids, although relatively weaker in bitterness intensity, play an indispensable role in the stability of the beer flavor and the durability of the foam. β-acids have the property of surface activity, and form a stabilizing effect with proteins in the beer, so that they can be used to stabilize the bitter taste. β-acids have surface-active properties, which enhance foam elasticity and durability by forming a stabilizing complex with proteins in the beer. Their mechanism of action is similar to that of a natural surfactant, which reduces the tension on the surface of the beer and enhances the stability of the foam, so that the beer always maintains a good appearance and a fine and rich foam layer during the drinking process. High-quality beers tend to have long-lasting and rich foam [*], which is largely due to the contribution of β-acids, providing consumers with a more pleasant drinking experience.

In addition to bittering substances, polyphenolic compounds in hops have a profound effect on beer flavor [*]. Polyphenols contain a variety of components such as flavonoids and phenolic acids, which actively participate in redox reactions during the beer brewing process and play a key role in the color formation of beer [*]. In the stages of malt saccharification and beer fermentation, polyphenols will react with other components, and with different degrees of reaction, the color of beer will show different shades from light yellow to dark brown. An appropriate amount of polyphenols can give beer a soft, mellow taste as well as a unique flavor, and at the same time, polyphenols have a certain antioxidant capacity, which can effectively inhibit the oxidation of lipids and the degradation of flavor substances in beer, which can help to prolong the shelf-life of beer and maintain the flavor stability of beer [*]. However, if the content of polyphenolic compounds in beer is too high, it may lead to excessive bitterness and astringency in beer, destroying the balance of beer taste and affecting the overall quality of beer [*].

1.3 Role of genes and enzymes in hops flavor formation

In the synthesis process of hops flavor substances, genes and enzymes play an extremely crucial role, which together constitute a complex and fine regulatory network. Modern biological research has shown that the synthesis of various flavor substances in hops is precisely regulated by a series of genes, which are like precise instruction codes encoding the synthesis of the corresponding enzymes, which act as biocatalysts to efficiently catalyze the synthesis of flavor substances and ensure the smooth progress of the whole synthesis process.

Coenzyme A ligase occupies a central position in the synthesis of secondary metabolites in hops and is closely linked to the synthesis pathways of α-acids, β-acids and polyphenolic compounds [*]. In terms of molecular biological mechanisms, coenzyme A ligases are able to activate the relevant substrates, making them more readily available for participation in the synthesis process in subsequent enzymatic reactions. Overexpression or knockout experiments of

coenzyme A ligase genes by advanced genetic engineering means, such as using CRISPR/Cas9 gene editing technology, can significantly alter the content and proportion of these flavor substances in hops, which in turn can have a significant impact on the flavor of beer. When the coenzyme A ligase gene is overexpressed, the intracellular content of the enzyme increases and the catalytic efficiency improves, which may promote the synthetic metabolic pathways of α-acids, β-acids and polyphenolic compounds, resulting in a more intense and mellow bitter taste and a richer and fuller flavor of the beer; conversely, if the gene is knocked out, the absence of the key enzyme for the synthesis of these flavor substances will result in the blockage of the corresponding synthetic reaction, and the flavor of the beer will become thin The flavor of the beer will become thin, single, and lose its original characteristics.

The expression of terpene synthase genes also has a crucial influence on the aroma composition of hops. Terpenoids are important components of hops aroma with lilac, rose aroma and apple aroma, which give beer its unique flavor [*]. The expression level of terpene synthase genes in hops cells directly determines the production of terpene synthase, which in turn affects the amount of terpene aroma substances synthesized. Overexpression of the terpene synthase gene through genetic engineering technology can increase the amount of terpene synthase synthesis, thereby enhancing the synthesis efficiency of terpene aroma substances, making the aroma of beer more intense and complex, and providing consumers with a more pleasurable olfactory experience [*]; on the contrary, if the gene is knocked out, the terpene synthesis pathway will be cut off, and the content will be decreased dramatically, and the aroma of the beer will become monotonous and boring. quality will also be reduced.

1.4 Coenzyme A ligase gene research progress

1.4.1 Gene family

Coenzyme A ligase genes belong to a large gene family widely present in plants, encoding enzymes that catalyze the ligation of various substrates to

coenzyme A, thereby playing a crucial role in the synthesis of secondary metabolites. These genes typically contain highly conserved domains, such as the AMP-binding domain and the coenzyme A ligase conserved motif DXDD. These conserved regions are prevalent across different species, indicating their significant biological functions during evolution [*].

The coenzyme A ligase gene family comprises numerous members that participate in diverse secondary metabolic pathways in various plants. For instance, in Arabidopsis thaliana, the At4CL1 gene encodes 4-coumarate-CoA ligase, which plays a key role in flavonoid synthesis [*]. Similarly, in hops (Humulus lupulus), members of the coenzyme A ligase gene family are involved in the synthesis of α-acids, β-acids, and polyphenolic compounds, which are essential components of beer flavor. These gene family members exhibit certain similarities in sequence and function but also possess unique functional and expression patterns.

Moreover, members of the coenzyme A ligase gene family display functional diversity across different plant species. For example, some members may specifically participate in the synthesis of a particular secondary metabolite, while others may function in multiple metabolic pathways. This functional diversity underscores the biological significance of the coenzyme A ligase gene family in plant adaptation to environmental changes and evolutionary processes.

1.4.2 Evolution and Phylogeny

Coenzyme A ligase genes are highly conserved in evolution, reflecting their critical role in plant secondary metabolism. Phylogenetic analysis reveals that coenzyme A ligase genes in hops cluster with homologous genes from other plants, indicating evolutionary conservation. This conservation is evident not only in gene sequences but also in their functions. Functional redundancy or division of labor among coenzyme A ligase gene family members may also influence the synthesis of flavor compounds. Phylogenetic analysis of coenzyme A ligase genes across different hop varieties can enhance our understanding of their evolutionary

conservation and diversity, providing a theoretical foundation for elucidating the mechanisms underlying hop flavor biosynthesis.

1.4.3 Expression Regulation Mechanisms

The expression of coenzyme A ligase genes is regulated by multiple factors, collectively modulating their activity and function, thereby influencing the synthesis of hop flavor compounds. Environmental factors, hormonal regulation, developmental stages, and genetic background all contribute to the expression of these genes. Environmental factors such as light and temperature significantly regulate the expression of coenzyme A ligase genes. Phytohormones like jasmonic acid also play a crucial role in their expression regulation [*]. The expression levels of coenzyme A ligase genes vary across different developmental stages of hops. Additionally, sequence and expression differences in coenzyme A ligase genes among hop varieties may lead to variations in flavor compound content and composition.

1.4.4 Metabolic Engineering Applications

Coenzyme A ligase genes hold broad potential for metabolic engineering and genetic improvement. Targeted regulation of their expression through gene editing technologies can optimize the synthesis of hop flavor compounds. For example, overexpression or knockout experiments using CRISPR/Cas9 gene editing can significantly alter the content and proportion of flavor compounds in hops, thereby profoundly impacting beer flavor [*]. Furthermore, promoter optimization or gene overexpression techniques can enhance the supply efficiency of acyl-CoA precursors, increasing the flux of bitter compound synthesis and providing novel strategies for hop genetic improvement [*].

1.5 Research Objectives and Significance

This study focuses on the Qingdao Large Flower hop (Humulus lupulus cv. Qingdaohua), a high-yield variety characterized by vigorous growth, extensive

vine development, and a long growth cycle. It thrives under high soil fertility and is well-suited for trellis cultivation [*]. Its flowers are cylindrical, resembling small pine cones, and exhibit a pale yellow-green or green color when fresh, darkening after drying [*]. Chemically, it contains balanced levels of α-acids and β-acids (α/β ratio: 1.0–1.5), with a total polyphenol content of approximately 7.0%. It is rich in hop oil components such as humulene and caryophyllene. In terms of flavor, it imparts a complex aroma profile, including floral, spicy, and herbal notes, contributing to a refreshing taste and fine foam in beer, with a smooth bitterness that harmonizes with malt aromas [*]. As a dual-purpose hop for both bitterness and aroma, it is suitable for brewing various beer styles, significantly enhancing their unique flavor and quality.

By cloning genes associated with flavor compound synthesis and conducting bioinformatics analyses, this study aims to elucidate their regulatory mechanisms in flavor formation and clarify their functional and evolutionary relationships. The findings will enrich the theory of plant secondary metabolism, provide a theoretical basis for hop molecular breeding, and offer a new model for studying secondary metabolic regulation in perennial herbaceous plants. This research will facilitate the breeding of high-quality hop varieties with elevated α-acid and terpene content, potentially shortening the breeding cycle, reducing traditional breeding costs, and promoting sustainable development in the beer industry. It holds significant value in both academic and industrial contexts.

Summary to Chapter 1

This chapter begins with an overview of the importance of hops (Humulus lupulus) as a key ingredient in beer brewing, emphasizing their central role in shaping the unique flavor of beer, stabilizing foam, and inhibiting the growth of miscellaneous bacteria. The study focuses on the "Qingdao Flower" hop variety, aiming to reveal the molecular mechanisms underlying hop flavor formation by cloning genes related to its flavor profile and conducting bioinformatics analysis. Specifically, the research centers on the coenzyme A ligase gene, which is closely

associated with the synthesis of α-acids, β-acids, and polyphenolic compounds, exploring its function in the formation of beer bitterness and aroma. Additionally, this chapter provides a detailed introduction to the historical background of hops and their developmental trajectory in the beer industry, highlighting their unique advantages as a natural ingredient. Through modern biotechnological approaches, such as CRISPR/Cas9 gene editing technology, overexpression or knockout experiments of target genes can significantly regulate the content and proportion of flavor compounds in hops, thereby optimizing the flavor characteristics of beer. This research not only provides theoretical support for the genetic improvement of hops but also lays a foundation for the optimization and advancement of beer brewing processes.

Chapter 2
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Experimental materials

2.1.1 Plant materials

Qingdao large-flowered hops plants were selected as experimental materials. During the peak growth period, healthy, pest-free leaves were selected for subsequent experiments. The collected leaves were quickly frozen and stored in liquid nitrogen to prevent RNA degradation and ensure the integrity and activity of the genetic material in the subsequent experiments.

2.1.2 Experimental reagents

TRIzol reagent, chloroform, isopropanol, 75% ethanol (DEPC water preparation), DEPC water, PrimeSTAR® Max DNA Polymerase, dNTP Mix, DL5000 DNA Marker, primers

2.1.3 Experimental Instruments

High-speed freezing centrifuge, mortar and pestle, pipette, RNase-free centrifuge tubes, PCR amplifier, constant temperature metal bath, gel electrophoresis system

2.2 Experimental Methods

2.2.1 RNA extraction

Take 100 mg of leaves in a pre-cooled mortar, add liquid nitrogen and grind until powdered and transfer to a 1.5 mL centrifuge tube. Add 1 mL of TRIzol reagent, vortex and mix well, and let it stand at room temperature for 5 min, then add 200 μL of chloroform, shake vigorously for 15 s, let it stand at room temperature for 2 min, and then centrifuge the supernatant at 12,000 rpm for 15 min at 4 ℃, take the supernatant into a new tube, add 500 μL of isopropanol,

invert the mixture, and then let it stand at room temperature for 10 min, and then centrifuge the supernatant at 12,000 rpm for 10 min at 4 ℃ again. After discarding the supernatant, the precipitate was washed with 1 mL of 75% ethanol, centrifuged at 7500 rpm for 5 min at 4°C, and the washing was repeated twice. Discard ethanol and dry the precipitate at room temperature, and finally add 50 μL of DEPC water to dissolve RNA.

2.2.2 Reverse transcription to obtain cDNA

The reverse transcription reaction system (20 μL) contained 2 μg of RNA template, 1 μL of 50 μM Oligo(dT)$_{18}$ primer, 1 μL of 50 μM Random Hexamers primer, 1 μL of 10 mM dNTP Mix, 1 μL of PrimeScript RT Enzyme Mix I and 4 μL of 5× PrimeScript Buffer. The reaction conditions were: incubation at 37°C for 15 min, inactivation at 85°C for 5 s, and the product was stored at -20°C for reserve.

2.2.3 Nested PCR amplification and comparison

The outer PCR reaction system (50 μL): 25 μL 2× PrimeSTAR Max Premix, 2 μL cDNA template, 2 μL 10 μM outer forward primer, 2 μL 10 μM outer reverse primer, and add ddH_2O to 50 μL. The reaction procedure was as follows: pre-denaturation at 95℃ for 5 min, denaturation at 95℃ for 30 s, annealing at 58℃ for 30 s, extension at 72℃ for 2 min, a total of 30 cycles. The reaction program: pre-denaturation at 95℃ for 5 min, denaturation at 95℃ for 30 s, annealing at 58℃ for 30 s, extension at 72℃ for 2 min, a total of 30 cycles; final extension at 72℃ for 10 min.

Take 1 μL of outer PCR product as template, add inner PCR system (50 μL): 25 μL 2×PrimeSTAR Max Premix, 1 μL of outer product, 2 μL of 10 μM inner forward primer, 2 μL of 10 μM inner reverse primer, and add ddH_2O to 50 μL. Reaction program: pre-denaturation at 95°C for 5 min; denaturation at 95°C for 30 s, annealing at 60°C for 30 s, final extension at 72°C for 10 min. 60°C

annealing for 30 s, 72°C extension for 1.5 min, a total of 35 cycles; 72°C final extension for 10 min.

The PCR products were detected by 1% agarose gel electrophoresis, and the target bands were cut and purified by gel recovery kit (Qiagen).

The purified PCR products were sent to DynaTech Biotechnology Co., Ltd. for double-end sequencing, and the resulting sequences were compared with the hops coenzyme A ligase gene in the NCBI database by BLAST.

2.3 Bioinformatics Analysis of Acetyl-CoA Synthetase Gene

2.3.1 Gene Sequence Alignment

The analysis was conducted using DNAstar software. First, the NCBI website was accessed, and the complete name "Humulus lupulus acetyl-coenzyme A synthetase, chloroplastic/glyoxysomal-like (LOC133816940), transcript variant X2, mRNA" along with its NCBI sequence number "XM_062249287.1" were entered. The published sequence in NCBI was then compared with the PCR-cloned product to analyze the differences in their CDS regions.

2.3.2 Prediction of Protein Higher-Order Structure

The SwissModel program (https://swissmodel.expasy.org) was utilized to predict the higher-order structure of the protein. DNAstar was employed to identify the open reading frame (ORF) in the gene sequence, and the nucleotide sequence corresponding to the ORF was translated into an amino acid sequence to obtain the primary structure of the protein. Further homologous sequence alignment and conserved domain analysis were performed using databases such as NCBI to verify sequence accuracy and identify key functional domains.

The TMHMM online tool (http://www.cbs.dtu.dk/services/TMHMM/) was used to input the amino acid sequence of the acetyl-CoA synthetase protein. Default parameters were applied to obtain the transmembrane propensity values at each site. Based on whether transmembrane helical domains (approximately

20–25 amino acids) with significantly higher propensity values (>0.5) and continuity were present, the protein was determined to be transmembrane or not.

Protein secondary structure prediction was conducted using SOPMA (https://npsa-prabi.ibcp.fr/). Accurate and complete FASTA-format amino acid or nucleotide sequences were retrieved from the database and pasted into the online platform (parameters could be adjusted as needed, with defaults being sufficient). After submitting the task and awaiting analysis completion, secondary structure types such as α-helices were extracted from the results report to analyze the proportion and distribution of amino acids in the sequence.

Hydrophilicity and hydrophobicity analysis of the enzyme protein was performed using ProtScale (https://web.expasy.org/protscale/). The FASTA-format amino acid sequence was pasted into the input field, and the default hydrophobicity scale (Hphob. / Kyte & Doolittle) was selected, with the sliding window size adjusted as needed. Based on the generated hydrophilicity/hydrophobicity plot, positive values indicated hydrophobicity, while negative values indicated hydrophilicity, allowing analysis of the hydrophilic/hydrophobic characteristics across different regions of the sequence.

Summary to Chapter 2

In this chapter, we have described the detailed experimental procedures for RNA extraction and subsequent PCR amplification of the acetyl-CoA synthetase gene from Humulus lupulus. The nested PCR approach was employed to enhance the specificity and yield of the target gene fragment. The PCR conditions, including primer concentrations, annealing temperatures, and cycle numbers, were carefully optimized to ensure the accurate amplification of the desired sequence. The amplified products were visualized through agarose gel electrophoresis, purified, and sent for sequencing to confirm their identity.

Furthermore, we have outlined the bioinformatics analysis performed on the cloned acetyl-CoA synthetase gene sequence. This included sequence alignment with the published sequence in NCBI to identify any differences in the CDS

regions, prediction of the protein higher-order structure using SwissModel, and analysis of the protein's transmembrane domains, secondary structure, and hydrophilic/hydrophobic properties. These analyses provided insights into the structural and functional characteristics of the acetyl-CoA synthetase protein, laying the foundation for further studies on its role in hop metabolism and potential applications in biotechnology.

Chapter 3
EXPERIMENTAL PART

3.1 RNA extraction

Total RNA was extracted from healthy leaves of Qingdao Dahlia hops using the TRIzol method, and was detected by 1% agarose gel electrophoresis, which showed clear 28S and 18S rRNA bands, and the brightness of the 28S bands was about two times that of the 18S bands, indicating that the integrity of the RNA was good (Figure 3-1). Measurement using NanoDrop 2000 showed that the OD_{260}/OD_{280} ratio was 1.91, and the RNA concentration was 1.18 μg/μL, which was in accordance with the requirements of the reverse transcription experiment.

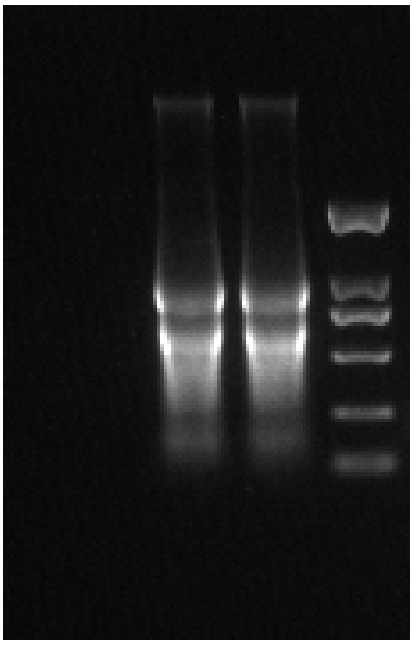

Figure 3-1 RNA extraction results

3.2 PCR amplification of coenzyme A ligase gene fragments

Coenzyme A ligase gene was amplified by nested PCR using cDNA synthesized by reverse transcription as template. The outer PCR product was detected by electrophoresis, showing a specific band at about 1650 bp, which was consistent with the expected fragment size; the inner PCR further enriched the target gene, and a clear band of about 1521 bp was obtained (Fig. 3-2), with no

obvious non-specific amplification, indicating that the target gene was successfully cloned.

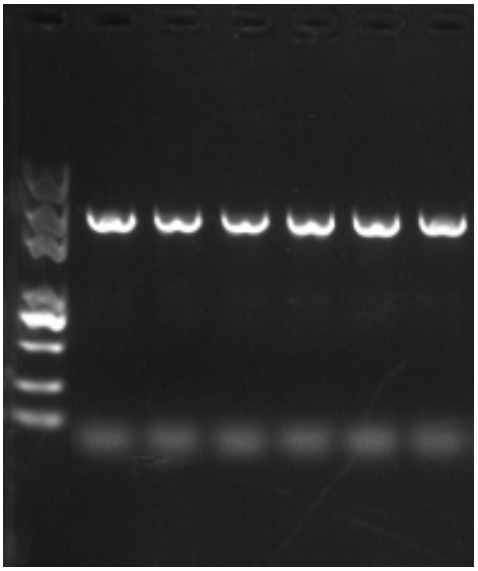

Figure 3-2 PCR amplification results

3.3 Analysis of Physicochemical Properties of Coenzyme A Ligase

Hydrophilicity and hydrophobicity analysis of the enzyme protein was conducted using ProtScale (https://web.expasy.org/protscale/).

3.4 Gene Sequence Sequencing and Alignment

After bidirectional sequencing of the PCR product, it was aligned with the sequences published in NCBI, showing 100% consistency. This gene is a protein-coding gene, and its protein product performs relatively fixed biological functions. To maintain functional stability, its structure and gene sequence are usually conserved.

atattaattg ccttaaatag aacacaaccc aaattaacaa aactccacaa tcaccacaaa
cgcttttgtt tatgtataat agtatcaaac attgctggcc aaactctccc ccaagctatc
attttcatgc aaattattaa aattatattt taatttaagt aaattgatga aatatggcat
tggaattagc ccacattttt tgctataaat tggtcaaagt tcaaactcgg gccagggccg
aagtcgcttc ttcactaatc tcaaaattca attcctatta ttccattaat aaaacaaaaa
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tcaatcatgt tcgcaattct tccggcgccg gaaaaatcca ccgtttgaac ggtgtcattc
tcggcgaggt attagcttct gaggaaaatg atctcgtctt tccaaccgat gaattctccc
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atagtgttta ctcggagaat ctcgatgtca gcaaaggaag agtcaacatt gagtggttca
agggaggtgt taccaacatt tgttacaact gtttggatag aaatgtggaa gctggacttg
gtgacaaagt tgccttgtac tgggaaggca ataacactgg atttgatgac tctttaactt
actttcaact tctccacaga gtttgccagc ttgcaaatta tttgaaagat atgggagttc
aaaagggtga tgctgttgtc atttacttac ccatgctatt ggaacttccc atcacaatgc
tagcatgtgc tcgcattggt gctgttcatt cggttgtttt cgccggagtt tctgcagaat
ctcttgctca gagaatcatt gattgcaaac caaaagttgt tgtcacttgc aatgctgtta
aaaggggtcc taagatcatt catctcaaag atatagtgga tgcggccctt gtggaatctg
ccaaaactgg catccccata gatgcatgcc ttgtctacga aaatcaattg gctatgaaga
gggaagttac taaattgcaa gatggaagag atgtatggtg gcaggacgtc attcctaaat
atccaactca ttgtgcagtg gagtgggttg atgctgagga tccattgttt ctgctataca
ctagtgggag cactggaaaa cctaaggggg ttctccatac aactggagga tatatggtgt
atactgcaac aacattcaaa tacgcatttg actataaacc gtctgatata tactggtgta
cagctgactg tggttggatt actgggcaca gctatgtcac atatggacca ctgctcaatg
gagcaacttc aatagttttt gaaggggctc ctaattatcc tgattcggga cgctgttggg
acatcgtaga taaatacaaa gttacaatat tttacactgc ccccacattg gtgcggtccc
tcatgcgtga tggtgatgag tatgtaattc gctactcaag aaaatcctta cggatccttg
gcagtgctgg tgaacctatc aatccaagtg catggaggtg gttttacaat gtggttggag
agtcaaggtg ccctatttct gatacatggt ggcaaactga aactggtggc ttcatgatta
ctccgttacc tggtgcctgg cctcagaagc ctggatctgc aacctttcct ttctttggag
ttaagcccgt tatagtagat gagaaaggtg ttgagattga aggggagtgc agtggatatt
tgtgtgtgaa aggctcttgg cctggagcgt tccgaaccct ctatggtgac catgagcaat
atgaaaccac ttactttaag cctttcactg gttattactt cactggtgat ggctgcagaa
gggacaagga tggataccac tggcttacag gaagagttga tgatgttata aatgtcagtg
gacatcgtat tggtacagca gaagttgaat ctgctctggt ttcacatccc aagtgtgcag
aagctgctgt ggttggtatt gagcatgagg tgagccttac agtgaagaac tacgaaagag
tctcatcctt actgtaagaa agcagatagg agcgtttgcc gcaccagaca ggatccactg
ggcgcctggg cttccaaaga caagaagtgg aaagattatg aggaggattc tgagaaaaat
tgcttctggt cagttagagg agcttggaga catgagcaca cttgcagacc cttatgtagt
cgaacagctc atttcacttt ccaattgctg acgacatttc t

Figure 3-3 Coenzyme A ligase gene sequence

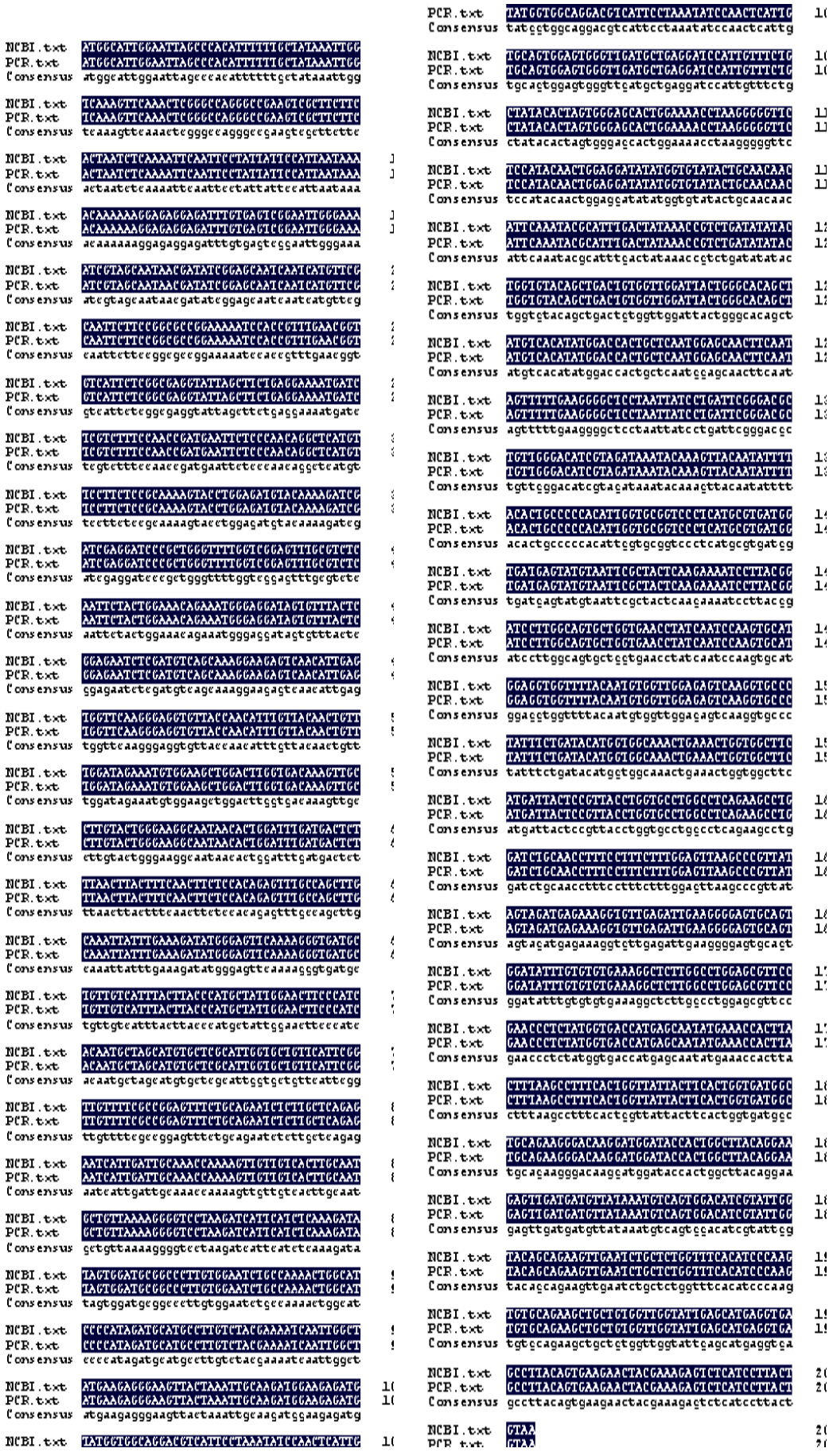

Figure 3-4 Sequence alignment results of coenzyme A ligase genes

3.5 Higher-Order Structure of Coenzyme A Ligase Protein

3.5.1 Primary Structure Analysis of the Protein

The open reading frame of the gene is 1,521 bp in length, encoding 507 amino acids. The amino acid sequence was obtained through translation using DNAstar software and verified via NCBI's BLASTp, showing 100% homology with the known amino acid sequence of hop coenzyme A ligase. The protein sequence contains a typical AMP-binding domain and the conserved DXDD motif of coenzyme A ligase (Figure 3-5). The AMP-binding domain serves as the core region for catalyzing the activation of substrates (such as isovaleric acid and 4-coumaric acid) to generate acyl-CoA precursors. The DXDD motif directly participates in coenzyme A binding and catalytic reactions, providing the structural basis for its involvement in the synthesis of α-acids, β-acids, and polyphenolic compounds.

MALELAHIFCYKLVKVQTRARAEVASSLISKFNSYYSINKTKKERRFVSRNWENRSNNDIGAINHVRNSSGA
GKIHRLNGVILGEVLASEENDLVFPTDEFSQQAHVPSPQKYLEMYKRSIEDPAGFWSEFASQFYWKQKWEDSVYSE
NLDVSKGRVNIEWFKGGVTNICYNCLDRNVEAGLGDKVALYWEGNNTGFDDSLTYFQLLHRVCQLANYLKDMGVQK
GDAVVIYLPMLLELPITMLACARIGAVHSVVFAGVSAESLAQRIIDCKPKVVVTCNAVKRGPKIIHLKDIVDAALV
ESAKTGIPIDACLVYENQLAMKREVTKLQDGRDVWWQDVIPKYPTHCAVEWVDAEDPLFLLYTSGSTGKPKGVLHT
TGGYMVYTATTFKYAFDYKPSDIYWCTADCGWITGHSYVTYGPLLNGATSIVFEGAPNYPDSGRCWDIVDKYKVTI
FYTAPTLVRSLMRDGDEYVIRYSRKSLRILGSAGEPINPSAWRWFYNVVGESRCPISDTWWQTETGGFMITPLPGA
WPQKPGSATFPFFGVKPVIVDEKGVEIEGECSGYLCVKGSWPGAFRTLYGDHEQYETTYFKPFTGYYFTGDGCRRD
KDGYHWLTGRVDDVINVSGHRIGTAEVESALVSHPKCAEAAVVGIEHEVSLTVKNYERVSSLL

Figure 3-5 Primary structure of protein

3.5.2 Transmembrane Structure Analysis

The transmembrane regions of the CoA ligase protein were analyzed using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). The results showed that the transmembrane propensity values for the entire amino acid sequence were below 0.5, and no transmembrane helical domains composed of 20-25 consecutive amino acids were detected (Figure 3-6). According to transmembrane structure prediction rules, when no region in the sequence exhibits a transmembrane propensity value significantly higher than the threshold (>0.5), the protein is classified as a non-transmembrane protein. It is inferred that this protein exists in a soluble form within the intracellular environment and can function without requiring transmembrane transport.

Further analysis was conducted using the protein secondary structure prediction website SOPMA (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html).

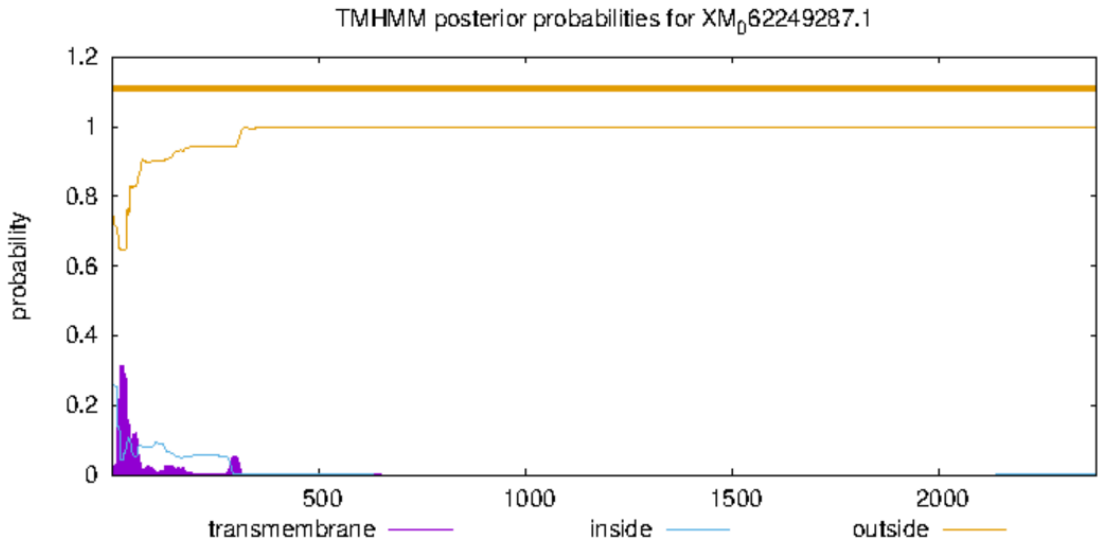

Figure 3-6 Transmembrane sites of the Coenzyme A ligase gene

3.5.3 Protease Hydrophilicity-Hydrophobicity Analysis

The hydrophilicity-hydrophobicity analysis of the enzyme protein conducted via ProtScale (https://web.expasy.org/protscale/) revealed that most regions of the coenzyme A ligase protein sequence exhibited values close to or below zero, indicating that the protein is predominantly hydrophilic, with only a few short sequence regions displaying weak hydrophobicity (Figure 3-7). The hydrophilic characteristics suggest that this protein likely exists primarily in aqueous environments such as the cytoplasm, facilitating its efficient binding with substrates within the cell and enabling catalytic functions.

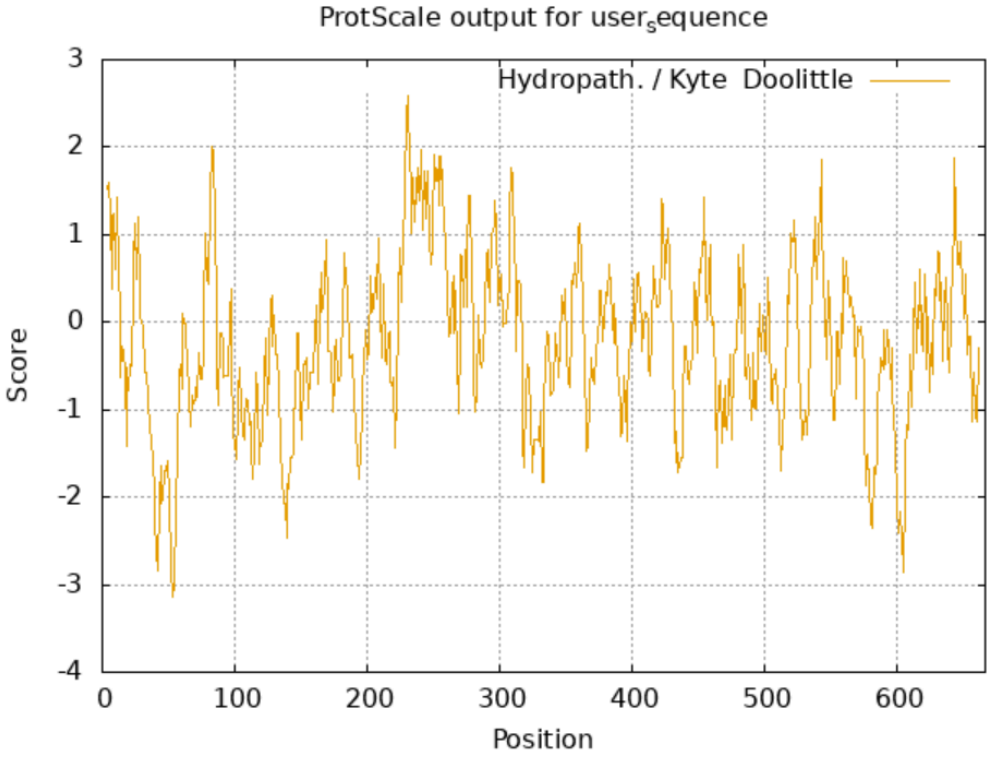

Figure 3-7 Hydrophilicity and hydrophobicity analysis of protease

3.5.4 Advanced Structure and Functional Prediction

The SwissModel program (https://swissmodel.expasy.org) was employed to predict the protein's advanced structure. Results demonstrated that it forms a typical α/β-fold structure containing multiple conserved functional domains (Figure 3-8), consistent with the characteristic spatial conformation of coenzyme A ligase. Analysis via the String-DB database revealed potential interactions between this protein and flavor compound synthesis-related enzymes such as terpene synthases, suggesting its cooperative catalytic role in the flavor compound synthesis network of hops.

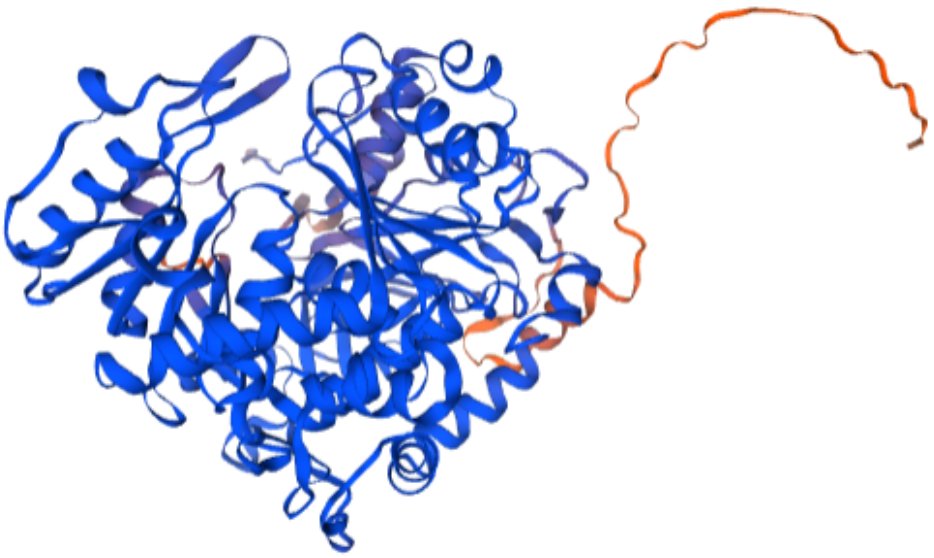

Figure 3-8 Advanced Protein Structure

The secondary structure of the protein was predicted using SOPMA (https://npsa-prabi.ibcp.fr/), revealing that α-helices account for 32.23% (215 amino acids), extended strands (β-sheets) for 17.69% (118 amino acids), and β-turns and random coils for approximately 50%. The α-helices and β-sheets form a stable structural framework (Figure 3-9), while the turns and coils confer flexibility to the protein. This structural combination facilitates the maintenance of conformational stability in the enzyme's active center while accommodating the conformational adjustments required for substrate binding.

The distribution of blue (α-helices), red (extended strands), green (β-turns), and purple (random coils) shows that α-helices and extended strands are alternately arranged, forming a typical α/β-fold structure, with turns and coils primarily located at domain junctions (Figure 3-10). These structural features align with the typical spatial conformation of CoA ligases, where α-helices and β-sheets constitute the core framework of the catalytic domain, while the turn and coil regions may participate in substrate recognition or interactions with other proteins, providing a structural basis for the enzyme's functional diversity.

```
SOPMA :
   Alpha helix       (Hh) :   215 is  32.23%
   3_10  helix       (Gg) :     0 is   0.00%
   Pi helix          (Ii) :     0 is   0.00%
   Beta bridge       (Bb) :     0 is   0.00%
   Extended strand   (Ee) :   118 is  17.69%
   Beta turn         (Tt) :     0 is   0.00%
   Bend region       (Ss) :     0 is   0.00%
   Random coil       (Cc) :   334 is  50.07%
   Ambiguous states  (?)  :     0 is   0.00%
   Other states           :     0 is   0.00%
```

Figure 3-9 Secondary structure types and amino acid proportions

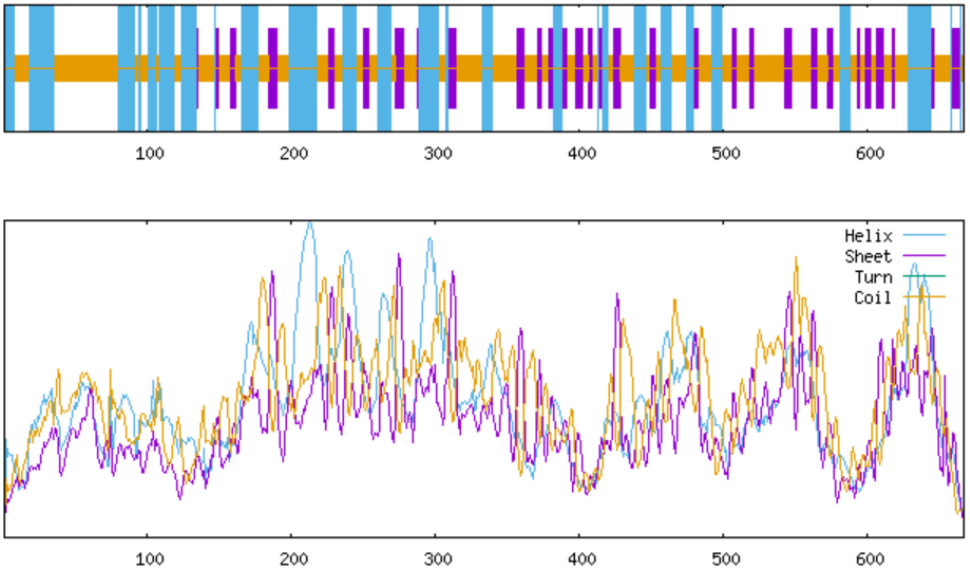

Figure 3-10 Protein Secondary Structure

Note: Blue (helix): α-helix, green (turn): β-turn, red (sheet): extended main chain, purple (coil): random coil.

3.6 Discussion and Prospects

This study focused on the cloning and analysis of the hop CoA ligase gene. Total RNA was successfully extracted from hop leaves using the TRIzol method, and cDNA was obtained through reverse transcription. The target gene was cloned via nested PCR, and its sequence characteristics and evolutionary status were elucidated through sequencing and bioinformatics analysis. The experimental results demonstrated that the cloned CoA ligase gene exhibited high sequence identity with homologous sequences in the NCBI database, and its encoded protein contained a typical AMP-binding domain and conserved CoA ligase motifs, suggesting its catalytic role in substrate activation during secondary metabolism. However, the current study only completed gene cloning and preliminary sequence analysis, without delving into its expression patterns in hops or its direct association with flavor compound biosynthesis.

3.6.1 Sequence Conservation and Functional Stability

The cloned hop CoA ligase gene showed 100% identity with homologous sequences in the NCBI database, and its encoded protein contained highly conserved AMP-binding domains and DXDD motifs (Figure 3-5). This feature aligns with the functional domain distribution of this gene family in plants such as *Arabidopsis*. The presence of conserved domains indicates that CoA ligase plays an irreplaceable core role in catalyzing the synthesis of acyl-CoA precursors, and its sequence stability is fundamental for maintaining the integrity of hop flavor compound biosynthesis pathways. Notably, the conservation of this gene across different hop varieties may correlate with the universality of flavor compound composition. For example, the balanced α/β-acid ratio (1.0–1.5) in Qingdao Da Hua hops might be directly linked to the enzyme's catalytic efficiency toward different substrates. Future studies could compare sequence variations of this gene among hops with distinct aroma profiles (e.g., floral or bitter types) to elucidate its intrinsic relationship with flavor phenotypic diversity.

3.6.2 Protein Structural Features and Catalytic Mechanisms

Hydrophilicity analysis revealed that the enzyme is predominantly hydrophilic (Figure 3-7) and lacks transmembrane domains (Figure 3-6), suggesting its soluble cytoplasmic localization. This characteristic, combined with its secondary structure—comprising α-helices (32.23%), β-sheets (17.69%), and flexible turns/coils (~50%)—determines its catalytic specificity. For instance, the α/β-fold pattern of the AMP-binding domain (Figure 3-8) provides a precise binding pocket for substrates (e.g., 4-coumaric acid), while the DXDD motif coordinates magnesium ions to catalyze CoA ligation. String-DB predicted potential interactions between this protein and terpene synthases (Figure 3-8), implying its possible role in co-regulating the biosynthesis of hop aroma and bitter compounds through metabolic networks. This offers new insights into the molecular mechanisms underlying "bitter-aroma balance."

3.6.3 Study Limitations and Future Directions

The limitations of this study lie in the lack of gene expression pattern analysis and functional validation in hops. Future research could employ real-time quantitative PCR to measure the gene's expression levels in different hop tissues (e.g., floral organs, stems) and developmental stages, correlating them with the content of flavor compounds such as α- and β-acids. Additionally, CRISPR/Cas9 gene editing could be used to generate overexpression or knockout hop plants to observe changes in flavor compound content, thereby clarifying the gene's specific role in flavor formation. Furthermore, integrating multi-omics (transcriptomics, metabolomics, etc.) approaches would comprehensively elucidate the metabolic networks and regulatory mechanisms involving the CoA ligase gene. This would provide deeper theoretical foundations for hop genetic improvement and beer brewing process optimization, advancing the beer industry toward precision and molecular design.

Summary to Chapter 3

This chapter summarized the key findings of the study on the hop CoA ligase gene. We successfully cloned the gene from hop leaves and conducted sequence analysis, revealing its high sequence identity with homologous sequences and the presence of conserved functional domains. The protein encoded by this gene was predicted to be localized in the cytoplasm and possess specific catalytic mechanisms. However, the study was limited to gene cloning and sequence analysis, without exploring gene expression patterns or functional validation. Future research directions were proposed, including gene expression analysis, functional validation using CRISPR/Cas9 gene editing, and multi-omics approaches to elucidate metabolic networks and regulatory mechanisms. These future studies will contribute to a deeper understanding of hop flavor compound biosynthesis and provide theoretical foundations for hop genetic improvement and beer brewing process optimization.

CONCLUSION

In summary, the study of the hop CoA ligase gene has shed light on its potential role in the biosynthesis of hop flavor compounds. The cloned gene exhibits high sequence identity with its homologs and contains conserved functional domains, indicating its core function in catalyzing the synthesis of acyl-CoA precursors. Despite the limitations of this study, such as the lack of gene expression pattern analysis and functional validation, the proposed future directions, including gene expression analysis, CRISPR/Cas9 gene editing for functional validation, and multi-omics approaches, promise to provide a deeper understanding of the metabolic networks and regulatory mechanisms involved in hop flavor compound biosynthesis. These future studies will not only advance our knowledge of hop genetics but also contribute to the optimization of beer brewing processes, ultimately driving the beer industry towards precision and molecular design.

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