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 CPN60 gene of grape molecular chaperone**

First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
Yufei LI

Scientific supervisor
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Reviewer
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Educational and professional program Biotechnology

APPROVE
Head of Department of Biotechnology, Leather and Fur, Professor,
Doctor of Technical Science
Olena MOKROUSOVA
_____
«___»_____2024

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Yufei LI

1. Thesis topic **Cloning and analysis of CPN60 gene of grape molecular chaperone**

scientific supervisor Tetiana Shcherbatiuk, Dr. Sc., Prof.

approved by the order of KNUTD "__"_____2024, №____

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

4. Date of issuance of the assignments_____

EXECUTION SCHEDULE

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	From 01 April 2024 to 11 April 2024	
2	Chapter 1. Literature review	From 06 April 2024 to 20 April 2024	
3	Chapter 2. Object, purpose, and methods of the study	From 21 April 2024 to 30 April 2024	
4	Chapter 3. Experimental part	From 01 May 2024 to 10 May 2024	
5	Conclusions	From 07 May 2024 to 12 May 2024	
6	Draw up a bachelor's thesis (final version)	From 20 May 2024 to 25 May 2024	
7	Submission of qualification work to the supervisor for feedback	From 25 May 2024 to 27 May 2024	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	27 May 2024	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	31 May 2024	
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	03 June 2024	

I am familiar with the task:

Student _____Yufei LI

Scientific supervisor _____Tetiana SHCHERBATIUK

SUMMARY

Cloning and analysis of CPN60 gene of grape molecular chaperone – Manuscript.

Qualification thesis on the specialty 162 «Biotechnology and Bioengineering». – Kyiv National University of Technologies and Design, Kyiv, 2024.

Grapes are widely cultivated fruit trees in the world, rich in antioxidant, anti-aging and cardiovascular disease prevention health function substances such as resveratrol and anthocyanins, and have a broad consumer market and huge economic value, and grape production is also restricted by various factors, so how to improve grape yield is also of great research significance. In all the life activities of grape, photosynthesis is closely related to the improvement of grape yield. Previous studies have shown that there is a molecular chaperone Cpn60 in plants that can use the hydrolysis of ATP to help new and formed or immature peptides complete the folding assembly, thus participating in the folding equipment process of proteins that play a key role in photosynthesis, and thus affecting the efficiency of photosynthesis. Therefore, in this study, CPN60 gene of different varieties of grapes was cloned, and amino acid sequence analysis of the protein encoded by this gene, protein structure prediction, protein physicochemical action analysis, protein interaction and exploration of its biological function were carried out.

Keywords: Grapes; Photosynthesis; CPN60; Cloning; Protein analysis.

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INTRODUCTION

Vitis vinifera. L is a widely cultivated fruit tree worldwide[1]**Помилка! Джерело посилання не знайдено.**, which is rich in beneficial substances such as resveratrol and anthocyanins for the body. It can effectively resist oxidation, anti-aging, and prevent cardiovascular diseases[3][4]. Not only can it be made into various wines with unique flavors, but it can also be used for processing grape seed oil, polyphenolic yogurt, fruit vinegar, canned and sun dried raisins, etc. It has a broad consumer market and huge economic value **Помилка! Джерело посилання не знайдено.**[5][6][7]. How to improve grape yield and quality has always been a hot topic in scientific research and practice.

CHAPTER 1

1.1 Classification and Structure of Cpn60

Although the amino acid sequence of a protein contains tertiary spatial structural information, peptide chains may still not fold properly in cells. For certain proteins, the probability of becoming natural functional molecules through spontaneous folding is extremely low, and they are more likely to form non natural molecules. This non natural protein often exposes the internal hydrophobic regions to the outside. When it spontaneously folds slowly or undergoes incorrect folding, these hydrophobic surfaces are more prone to aggregation due to their exposure. When a small number of molecules begin to aggregate reversibly, this process may gradually evolve into irreversible aggregation. Once proteins undergo irreversible aggregation, they not only interfere with the normal structure and function of cells, but may even lead to cell death. Therefore, in order to maintain the stability and health of cells, they must have an efficient error correction system. In this system, heat shock proteins such as molecular chaperones are produced under specific stimuli and play a crucial role. Molecular chaperones, also known as chaperones, are a class of proteins that have no correlation in sequence but share common functions. They only participate in the assembly process of other peptide containing structures, helping them to detach on their own after proper assembly and not participate in the structure of these proteins when performing functions. Chaperonin is a molecular chaperone with a cavity structure that can encapsulate unfolded or misfolded proteins inside, utilizing ATP energy to provide an environment conducive to protein folding. Two types of chaperones have been discovered, type I chaperones, present in bacteria (GroEL), plastids (Cpn60), and mitochondria (Hsp60) [8]. Type II chaperones are mainly distributed in the cytoplasm of archaea and eukaryotic cells (i.e. CCT/TriC).[10] Unlike type I chaperones that rely on GroES/cpn10 to achieve tight substrate binding during protein folding, type II chaperones have a hat like structure that can self perform the

functions of GroES/cpn10[10][11]. The coding of type I chaperone proteins comes from multiple genes, and these genes exhibit highly conserved characteristics in sequence [12]. In addition, detailed research has been conducted on the Escherichia coli companion protein (GroEL), which is a typical representative of this protein family. These studies have revealed its protein folding function based on oligomeric protein structure and confirmed that it has a large number of folding chaperones [13][14].

The architecture of GroEL consists of 14 identical subunits precisely combined to form a cylindrical oligomeric structure. This cylinder is essentially derived from the nested structure of two heptamer rings. Each heptamer ring structure constructs an independent and enclosed chamber, which ensures the isolation of protein intermediates that fold within this space from the external environment, thereby preventing unexpected aggregation or hydrolysis and ensuring the smooth progress of protein folding process (Figure 1.1). The GroES helper chaperone protein appears as a circular structure composed of seven 10kD subunits, resembling a brim and covering one end of the cylindrical structure, providing a closed and stable chamber environment for protein folding [15][16]. At the other end of the cylinder, the circular structure undergoes conformational changes by hydrolyzing ATP, triggering the detachment of the GroES cap and releasing the folded protein [17].

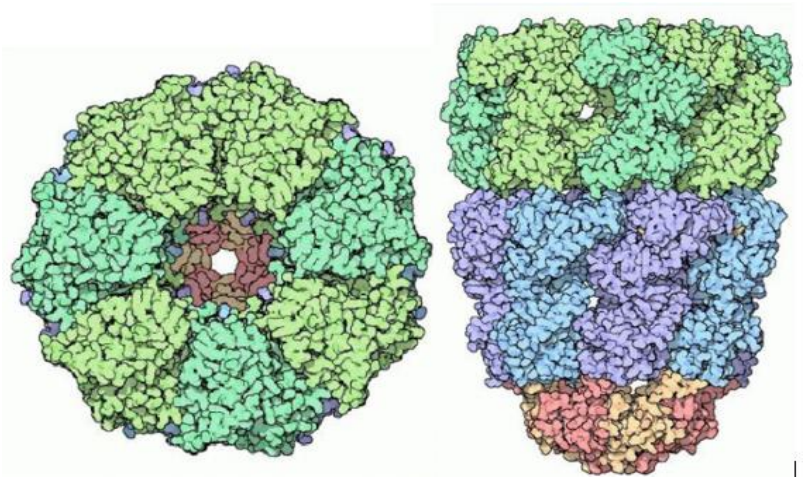


Figure 1.1Escherichia coli GroEL GroES complex

(Image source:https://pdb101.rcsb.org/motm/32)

Photosynthesis is a crucial process in which plants convert light energy into chemical energy, which is then stored in organic matter. In recent years, research has revealed the importance of a class of chaperone proteins in photosynthesis, which have attracted attention due to their involvement in the folding and assembly processes of key photosynthetic proteins such as Rubisco, NDH complexes, and Rubisco activating enzymes [18][8][19][20][21][22][23]. This type of chaperone protein is biologically known as chaperones and is widely present in prokaryotes and eukaryotes. Due to its molecular weight of about 60kD, it is often referred to as Chaperon60 (Cpn60). Cpn60 assists newly formed or immature peptides in their folding and assembly processes through the hydrolysis of ATP, providing strong support for the smooth progress of photosynthesis[18][19].

However, a series of experimental results have revealed significant differences between the crystal structure of Cpn60 in the plastid and the formation mode of its oligomers compared to GroEL[16]. Unlike GroEL, which consists of 14 identical subunits, Cpn60 in the plastid contains two distinct subtypes, namely alpha and beta [21][22][24]. These two subtypes exhibit a high degree of similarity, with 50% of the sequences being consistent. This similarity indicates a close evolutionary connection between them. Although the cpn60 gene in plastids is generally believed to be an evolutionary product of GroEL-1 in blue-green algae, both Rubisco subtypes have evolved into two or more paralogous forms in different higher plants. For example, in Physcomitrella patens, The subunit composition of Cpn60 presents a combination of 3 alpha subunits and 4 beta subunits [25], while in Brassica napus, this combination becomes 3 alpha subunits and only 1 beta subunit [26], while Arabidopsis has 2 alpha subunits and 4 beta subunits[27]. In addition, in rice (Oryza sativa), The subunit types of Cpn60 include three alpha subunits and three beta subunits [23].

Since its discovery, Cpn60 has been present in chloroplasts α And Cpn60 β The oligomers of companion proteins are considered to be their natural active state. There is a lot of evidence to support this theory, as oligomers of chaperone proteins isolated from a large number of species are composed of an equal amount of Cpn60

α And Cpn60 β Heteropolymers composed of subunits [22][28]. Moreover, in spinach (Spinacia oleracea), Cpn60 α And Cpn60 β Each specific antibody can always precipitate together with two subtypes [22]. Cpn60 extracted from peas (Pisum salivum) α And Cpn60 β Monomers can also form Cpn60 during in vitro reconstruction α β Heteropolymers[29].

Although the Cpn60 β of Brassica napus can form functional homologous oligomers after expression in E. coli and correctly fold the large subunit of blue-green algae Rubisco, it is surprising that Cpn60 α expressed in the same environment cannot be assembled into a tetramer alone and lacks corresponding functional activity [30]. The in vitro research results of pea Cpn60 and rapeseed Cpn60 are consistent, both indicating that Cpn60 β can successfully reconstruct and form active homologous oligomers in vitro. Under the presence of Cpn60 α , Cpn60 α β heteromers will preferentially form instead of Cpn60 β homooligomers. This discovery prompts us to speculate that heterooligomers may play a more important role in physiological mechanisms [29]. These oligomers play different roles within the plant body and play important biological roles.

In plant cells, The existence forms of Cpn60 protein are diverse. In addition to the common Cpn60a β heterodimerization, it can also independently form homologous oligomers with specific functions based on the Cpn60p subunit as the basic unit. In addition, a small number of specific Cpn60 subunits in chloroplasts can regulate substrate specificity in the oligomer environment, which suggests that there may be a variety of active chaperones with different functions in chloroplasts. This significant diversity, combined with different oligomeric structures of chaperone cofactors, greatly enriches the combination patterns of chaperone complexes, endowing them with high flexibility in regulatory effects and specificity, further expanding their potential applications in multiple fields [20].

1.2Research progress of Cpn60

The companion protein GroEL of Escherichia coli, as a representative of the Type I protein family, has been extensively studied in terms of structure and function. However, the homologous higher plant chloroplast Cpn60 protein, although structurally similar to GroEL, is relatively lacking in depth of research, which provides us with a broad space to further explore its characteristics and functions.

In the field of botany, preliminary research on Cpn60 originated from the chloroplasts of peas, where Cpn60 was identified as an oligomeric protein that binds to the initial rubisco large subunit (rbcL) and participates in the assembly of rbcL folding into mature rubisco whole enzymes [31]. Further research has shown that when antagonists against chaperone proteins are added, rbcL cannot be successfully assembled into a complete enzyme, emphasizing the crucial role of Cpn60 in rubisco folding processing[32][33]. The latest study on maize (Zea mays) also confirmed this discovery, revealing that maize rbcL can form complexes with Cpn60 subunits[34].

In Arabidopsis, there are 2 Cpn60 alpha subunits, 4 Cpn60 beta subunits, and 3 chaperone cofactors[35]. In recent years, scholars have conducted many exploratory studies on the functions of each subunit. The AtCpn60 α mutant obtained through T-DNA insertion mutation exhibits a phenotype of embryonic lethality. Research has found that the loss of Cpn60 α protein function greatly impairs chloroplast development, leading to poor embryonic development and inability to survive [36]. Another Arabidopsis mutant lacking Cpn60 β 1, Atcpn60 β 1, exhibits leaf cell death under short day conditions and can lead to seedling death in severe cases [37]. The dual mutants of cpn60 β 1 and cpn60 β 2 can also exhibit lethal phenotypes[25]. Currently, it is believed that, Cpn60 α l and Cpn60 β 1, Cpn60 β 2 can fold multiple functional proteins and exercise the function of housekeeping proteins, affecting the growth and development of Arabidopsis. Another Arabidopsis mutant lacking Cpn60 β 4, Atcpn60 β 4, exhibits defects in chloroplast NDH electron transfer function, The Atcpn60 β 4 mutant has almost no difference in appearance

from the wild-type, but during fluorescence detection, it was found that there is a functional deficiency in the NDH complex. Later research has shown that the mutation of cpn60 β 4 does indeed cause a specific subunit of NDH, The inability of NdhH to fold incense properly indicates that Cpn60 β 4 is necessary for the folding of NdhH [23]. In addition, when the cpn60 α 1 gene was mutated, Arabidopsis plants showed significant dwarfism, underdevelopment, and loss of NDH electron transfer function. Protein analysis also showed a significant reduction in the content of many chloroplast related proteins, indicating that the substrate proteins of Cpn60 α l are very rich and important, including NDH complex related proteins [23]. The study by Salvucci et al. (2008) revealed an interesting finding: when Arabidopsis faces heat stress, the chaperone protein β subunit (cpn60 β) in chloroplasts binds to RCA (Rubisco activator) through affinity chromatography. This combination may be necessary for plants to resist heat stress, prevent RCA from becoming inactive due to high temperatures, and ensure the maintenance of Rubisco activity, achieving thermal adaptation for photosynthesis. This discovery highlights the extensive role of Cpn60 protein as a chaperone protein, which involves diverse and functionally critical protein substrates and is of great significance for plant growth and adaptation to the environment.

Although there have been many studies on the Cpn60 protein in Arabidopsis, there are not many studies related to it in rice. Rice has three types of subunits and three types β Subgene[23], i.e. 0sCpn60 α 1 (0s12g17910), 0sCpn60 α 2 (0s03g64210), 0sCpn60 α 3 (0s09g38980), 0sCpn60 β 1 (0s06g02380), 0sCpn60 β 2 (0s02g01280) and OsCpn60 β 3 (ChrSy. fgenesh. gene. 28). There is 60-86% consistency in the primary structure between the three A subunits, with three β There is 61-84% consistency between subunits. Research shows that 0sCpn60 β The expression level of 1 is the highest, followed by 0sCpn60 α 1. 0sCpn60 α 2. 0sCpn60 β 2 and 0sCpn60 β 3. The least expressed is OsCpn60 α 3. OsCpn60 α

2 and OsCpn60 β 2 is mainly expressed during seedling growth, while others are more expressed within flag leaves[38].

Using T-DNA insertion technology, we successfully screened OsCpn60 α The mutant strains of L exhibit significant chlorosis characteristics during the seedling stage. Through in-depth genetic analysis, we have determined that this chlorotic phenotype originates from a recessive mutation at a single gene locus, and accurately identified the mutation locus as OsCpn60 α L genes. Further observation revealed that after the mutant plant reached the fourth leaf, the development of new leaves immediately stopped, ultimately leading to the death of the entire plant. In order to accurately verify the cause of this phenotype, we conducted a response experiment, and the experimental results clearly showed that the chlorosis phenomenon of the mutant leaves was indeed caused by OsCpn60 α Caused by the loss of genes. Subsequently, we used specific antibodies to detect the mutant and found that only the content of rbcL protein significantly decreased in the mutant, strongly suggesting OsCpn60 α L plays an indispensable role in the correct folding process of rbcL protein. This is also consistent with the function of Cpn60 discovered in other plants earlier[38].

1.3Mechanism of action of Cpn60

Proteins may experience misfolding and aggregation during synthesis and folding. To address this misfolding phenomenon, cells have evolved a molecular chaperone network as part of the protein homeostasis system, which can help proteins refold and maintain the natural conformation of mature proteins[39][40][41][42]. The molecular structure and mechanism of action of GroEL/GroES in type I molecular chaperones have been extensively studied. Taking GroEL/GroES as an example, as molecular chaperones, they exhibit unique molecular mechanisms in helping substrate proteins fold and assemble. During this process, there are two main views

on how the GroEL ring (i.e. oligomeric ring) interacts with the substrate. One pattern is that the upper oligomeric ring on the molecular chaperone structure is responsible for folding the substrate, while the lower ring is not involved in this process. The other mode shows that the upper and lower rings of the molecular chaperone structure can work together at the same time to fold and assemble the substrate (Figure 1.2).

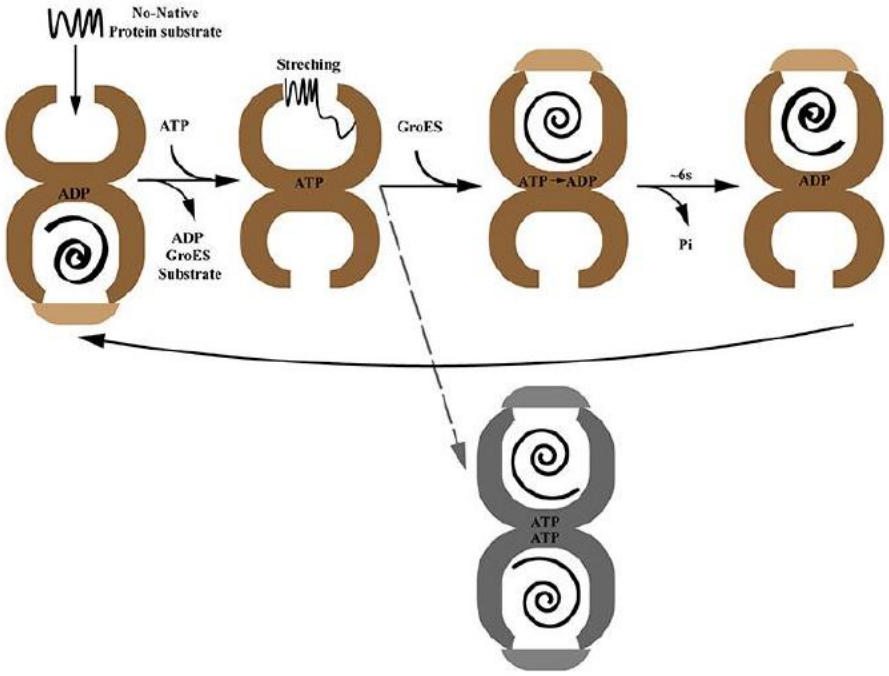


Figure 1.2 The mechanism of action of molecular chaperones
(Image source: Chloroplast Chaperonin: An Intricate Protein Folding Machine for Photosynthesis)

In its initial state, The GroEL subunit is located between the T state (low affinity for ATP) and the R state (high affinity for ATP) [16]. The open loop in the protein folding state captures polypeptide chains, and this interaction involves 3 or 4 GroEL domains. The next step is to bind with ATP, and the substrate protein stretches through conformational changes in the top domain, leading to the unfolding of misfolded protein intermediates [13]. Then GroES binds to the substrate occupied by ATP on the GroEL ring, known as the cis ring ternary structure, which triggers the top domain. The folding time of the hydrophilic chamber in this step depends on the ATP hydrolysis rate, which takes 6 seconds at 25 ℃[43]. Subsequently, the binding of

ATP in the opposite trans loop leads to the dissociation of GroES, as well as the release of substrate proteins and ADP. Meanwhile, the opposite trans ring becomes a new folding active cis ring [16][44]. For substrate proteins that are too large to fold (usually exceeding 60 kD), GroEL/GroES can still help them fold by binding and releasing from the trans loop [13][45].

Conclusions to chapter 1

From this, it can be seen that Cpn60 protein, as a protein with diverse composition and function, cloning the Cpn60 gene, studying its gene expression, protein structure and function, etc., can help to better understand the progress of various life processes and gain a deeper understanding of its role in living organisms.

CHAPTER 2
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Plant materials

In this experiment, a total of 8 grape varieties including Creasen seedless, Carmel, Merlot, Chardonnay, Syrah, and grape rootstocks 1103P, 5BB, and 140R were selected as experimental materials.

2.2 Reagent

The Total RNA extraction kit was purchased from Beijing Baiaoleibo Technology Co., Ltd., the column gel recovery kit was purchased from Shanghai Huzhen Industrial Co., Ltd., and the Taq DNA polymerase was purchased from Shanghai Yinjia Biopharmaceutical Technology Co., Ltd.

2.3 Extraction and reverse transcription of leaf RNA

The Total RNA extraction kit was used to extract RNA from grape leaves, and the specific process is as follows:

Weigh 2g of the extracted sample frozen at ultra-low temperature and quickly transfer it to a mortar pre cooled with liquid nitrogen. Grind the tissue with a pestle and continuously add liquid nitrogen until it is ground into powder;

Add 1ml RNAiso PIus to the mortar, completely cover the powdered sample, then let it stand at room temperature until the sample is completely melted, and continue grinding with a pestle until the cracking liquid becomes transparent;

Transfer the homogenate to a centrifuge tube and let it stand at room temperature for 5 minutes;

Centrifuge 12000g at 4 $^{\circ}$ C for 5 minutes, carefully aspirate the supernatant and transfer it into a new centrifuge tube;

Add chloroform (1/5 volume of RNAiso Plus) to the homogenate cracking solution, cover the centrifuge tube tightly, and shake vigorously by hand for 15

seconds. After the solution is fully emulsified (without phase separation), let it stand at room temperature for 5 minutes;

Centrifuge 12000g at 4 ° C for 15 minutes, carefully remove the centrifuge tube from the centrifuge, and transfer the supernatant to another new centrifuge tube;

Add an equal volume of isopropanol to the supernatant, invert the centrifuge tube and mix thoroughly, then let it stand at 15-30 ° C for 10 minutes;

Centrifuge 12000g at 4 ° C for 10 minutes, carefully discard the supernatant, slowly add 75% ethanol Iml along the centrifuge tube wall, gently invert and wash the centrifuge tube wall. Centrifuge 12000g at 4 ° C for 5 minutes, then carefully discard the ethanol;

Dry the precipitate at room temperature for 2-5 minutes, add an appropriate amount of RNase free water to dissolve the precipitate, and if necessary, gently blow the precipitate with a pipette. After the RNA precipitate is completely dissolved, store it at -80 ° C.

Use a UV spectrophotometer or fluorescence quantification method to determine the concentration and purity of RNA. The integrity of RNA was checked by gel electrophoresis.

Use the TAkara reagent kit to reverse transcribe the extracted RNA into cDNA. Prepare a reaction mixture on ice according to the following ingredients, gently blow and mix with a pipette, and react at 42 ℃ for 2 minutes.

reagent	amount of usage
5*gDNA Eraser Buffer	2.0μl
g DAN Eraser	1.0μl
TotalRNA	

RNase Free dH_2O	Up to 10μl

2.4 PCR amplification identification

Design primers based on the CDS of the grape companion protein CPN60 (LOC100244356) gene sequence downloaded from NCBI. The sequence is as follows, with a length of 1728 bp in the CDS region.

taaacccgtagagcctgaattgtgtaaaaccctaaaccctaaagactcctgctccttctagactcttccgttctctctaaaaggtgttgcttccctctcctcacgtcgcaaccg
agttctcctctctctctctctctgaaaccctcattttctctctctaaaaccccttcatttcgtgaagttacagcttctgcaactccccagccatgtaccgtttcgccaccagtctcgct
tccaaggctcggatcgctaggaacagcacccatcagattggtagtaggctgaactggagcagaaactatgctgctaaagacatcagattcggggttgaggctcgagccttg
atgcttaagggcgtggaagagctcgcagatgctgttagagtcaccatgggccccaaggggcgtaacgtggtactagagcaaagttttggtgccccaaaagtgacaaaaga
tggtgtaactgttgcaaaaagcattgagttcaaggatcgaatcaagaatgttggtgcaagccttgtgaaacaggttgcaaatgccactaatgatgtagcaggagatggtac
cacctgtgccacagtccttacccgagcaatatttactgaagggtgcaagtcggttgctgctggaatgaatgcaatggacctaagaaggggtatctcgatggcagtagatgct
gtagtgacaaacttgaaaagcagagctaagatgatcagcacatctgaagaaatagcacaggttgggacaatatcagcaaatggtgagagagaaattggtgaattgatcg
caaaggctatggagaaagttggcaaagagggagtaatcacaatttcagatgggaagacattgtataatgaattggaagttgttgaggggatgaagctggatagaggttac
atatccccttattttataacaaaccagaaaacccaaaaatgtgaactagaaaatcctcttgttctaatccatgagaagaaaatctcgaatatacatgctgttgtgaaagtgct
agagatggcattaaagactcagaggcctttactgattgttgctgaagatgtggaaagcgaagcactggccactcttattctaaataagcttcgtgctggaatcaaggtctgt
gccatcaaagcacctgggtttggagagaacaggaaggccgccctgcaggatctggcaactctcacaggaggggagcttataacggaagagcttggtttgaaccttgagaa
ggcagaactggatatgcttggcacttgcaaaaaggttacaatatctaaggacgatactgttatccttgatggggctggtgacaagaaggccattgaggaacggtgtgagca
gattaggtcagccattgaattgagcacttctgattatgacaaggaaaagttgcaagaaaggttggccaagctttctggtggtgttgctgtcttgaagataggaggagctagt
gaagctgaagttagtgagaagaaagatagggttacagatgctctaaatgctactaaggcagctgtagaagaaggaattgtaccaggtggtggcgtggcacttctttatgct
tccaaagagttggacaaactgccaacatcaaactttgatcagaagattggtgttcagattatccagaatgctctgaaaacacctgtgttcacaattgcttctaatgctggagt
tgaggggctgttgtggttgggaagctgttggagcaggataaccctgatcttggatatgatgcggccaaaggtgaatatgttgatatggtcaaagctggaattattgatcca
ctgaaagtaattagaacagctttggttgatgctgcaagtgtgtcatcgttgatgacaacaactgaagctgttgtggttgaactgccaaaggatgagaaggaagttcctgcaa
tgggaggtggcatgggtggcatggactactaacgggcctcgcagctggtagactgatctacctgacataacattatttccacccaaaaaattcacatctgaaatgagcatta
ttagggtttttgtcttgaatgaatgagcctttgtttttcctgtcataggtaaggcaaccatgtaatcatagtgagaggtcagtacttaggttctgataatgggggtgaaactgtc
actgatagtgcttggaaggatttgagacttcaatttttctcaaataaaaaattctcttccgtgca

Using cDNA as a template, perform nested PCR using the primers designed below, and clone to obtain the full length of CDS.

F-OUT: TAAACCCGTAGAGCCTGAAT

F-IN: CCCTAAAGACTCCTGCTCCT

R-IN: AAGGCTCATTCATTCAAGACAA

R-OUT: ATGATTACATGGTTGCCTTA

PCR amplification was performed using the designed primers. The PCR reaction system and conditions are as follows: the outer primers were used for the

first step of PCR amplification, and the PCR amplification program was pre denatured at 95 ° C for 7 minutes; 95 ° C denaturation for 30 seconds, 55 ° C annealing for 30 seconds, 72 ° C extension for 45 seconds, 30 cycles;

Extend at 72 ° C for another 7 minutes. After the first step of PCR amplification is completed, dilute the PCR product by 20 times and take 1 μ L is used as the amplification template for the second step of PCR, and then the inner primer is used for the second step of PCR amplification. The PCR amplification system is the same as the conventional PCR detection system, and the amplification program is the same as the conventional detection except for the cycle number being changed to 30.

Take 5 PCR products from each of the above μ L was electrophoretic in 2% agarose gel for 1h (5 V · cm^{-1}).

2.5Agarose gel electrophoresis and gel recovery

Using Shanghai Huzhen Industrial Co., Ltd.'s adhesive recovery reagent kit, the specific operation is as follows:

(1) The PCR products were dabbed into 1% agarose gel and put into the swimming pool for detection. After electrophoresis, cut the gene containing the target band under a UV lamp and place it in a centrifuge tube.

(2) Weigh the cut gel, add the same volume of Binching Buffer in the proportion of 0.1g: 0.1ml, and take a water bath at 55 ℃~65 ℃ for 5-10 minutes, during which, mix it every 2-3 minutes.

(3) Transfer to HiBincl Tm DNA column, centrifuge at 1000 rpm for 1 minute, and discard the filtrate.

(4) Add 300 to the adsorption column μ L Binding Buffer to HiBincl Tm DNA column at 1000 rpm

Centrifuge for 1 minute and pour out the filtrate.

(5) Add 700 to the adsorption column μ L SPW Wash Buffer (check if ethanol was added in advance) was added to a HiBincl Tm DNA column, centrifuged at 1000 rpm for 1 minute, and the filtrate was discarded.

(6) Repeat step (5).

(7) Centrifuge the column at 1000 rpm for 1 minute and pour out the filtrate.

(8) Put into a new 1.5 ml centrifuge tube and add 30-50 μ LRNase free ddH2O, centrifuge at 1000 rpm for 1 minute. Store at -20 ℃. Then observe and take photos with gel imaging system.

2.6Informatics analysis

Perform multiple sequence alignment of the CPN60 gene using DNAMAN software; Using SOPMA and SWISS-MODEL online software to predict and analyze the secondary and tertiary structures of proteins (NPS @: SOPMA secondary structure prediction (ibcp. fr) (SWISS-MODEL (explain. org)); Utilizing UNIPROT （ https://www.uniprot.org/uniprotkb ） Analyze the function of proteins; Utilizing STRING （ https://cn.string-db.org/ ） Tool analysis of interactions between molecular chaperone CPN60 and other proteins.

Conclusions to chapter 2

Through the extraction and reverse transcription of leaf RNA, as well as PCR amplification and identification, we can obtain the target gene for this experiment. This experiment used nested PCR, which has the characteristics of high sensitivity and specificity through two rounds of PCR reaction. During the PCR process, attention should also be paid to controlling the temperature at each step.

CHAPTER 3
EXPERIMENTAL PART

3.1Nucleotide sequence analysis of CPN60 gene

A specific band with a fragment length of about 1728bp was obtained through nested PCR amplification,PCR electrophoresis detection as shown in Figure 3.1, The Marker is DL2000.

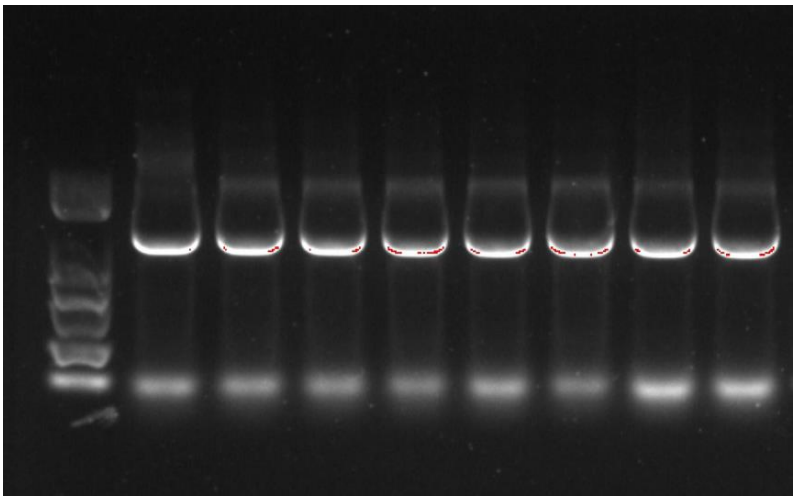
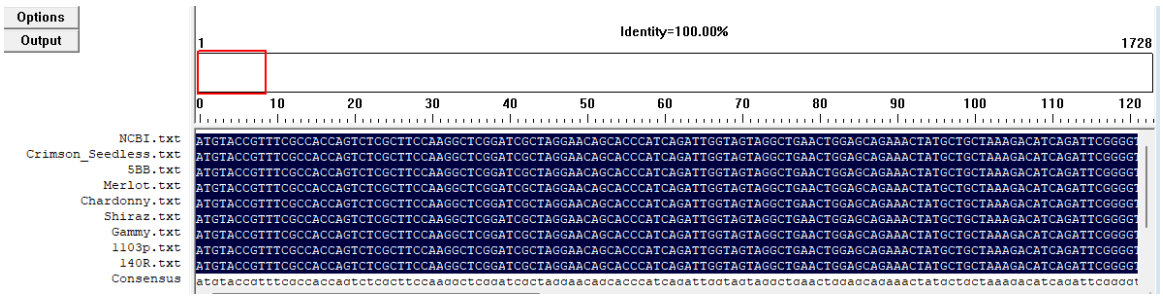


Figure 3.1 PCR electrophoresis detection

Multiple sequence alignment was performed using DNAMAN software, and the results showed that the amplified fragments of the CPN60 gene from different grape varieties had 100% sequence similarity (Figure 3.2). This may be closely related to the involvement of CPN60 in maintaining the structure of proteins related to photosynthesis, and its mutations or deletions can easily lead to plant death.



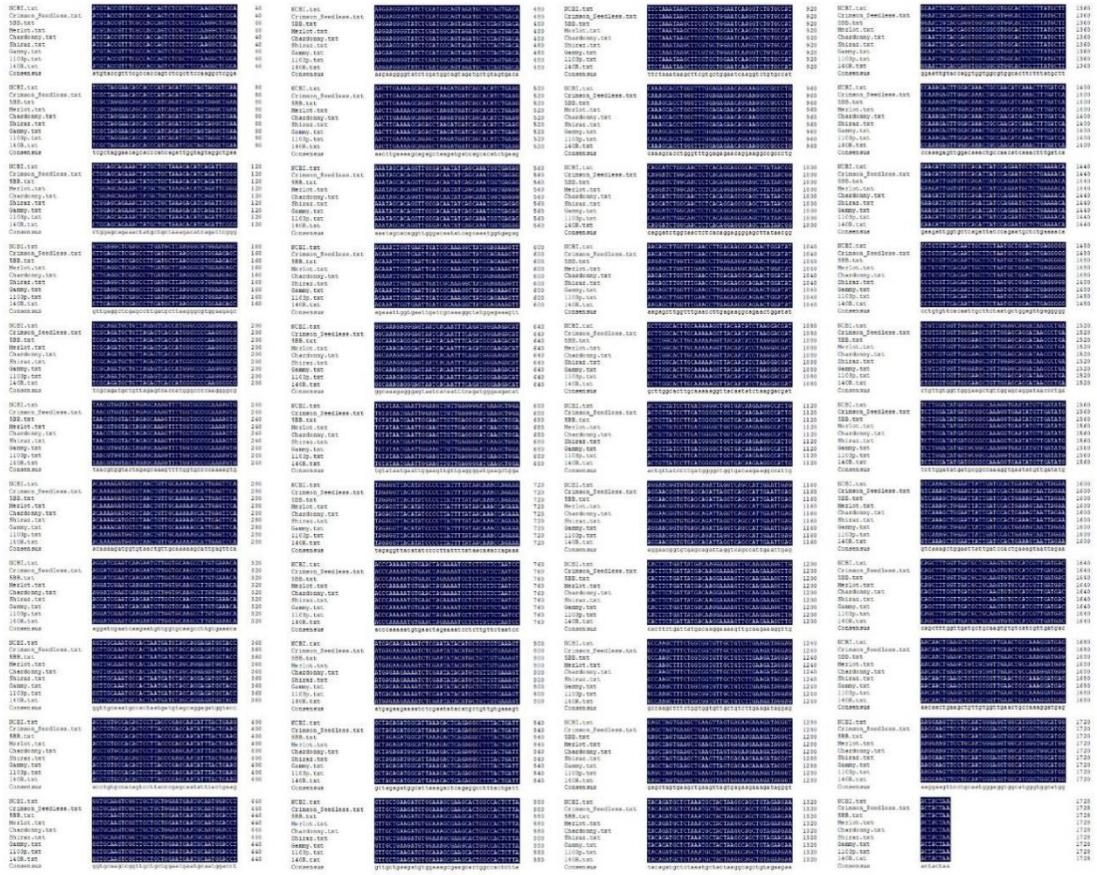


Figure 3.2 Multiple sequence alignment analysis

3.2 Prediction of CPN60 protein structure and function

Using ExPASy's ProtParam software to predict and analyze the physicochemical properties of grape CPN60 protein, it was found that the grape CPN60 gene encodes a total of 575 amino acids (Figure 3.3); The theoretical isoelectric point is 5.85, indicating an acidic protein; The molecular weight is 61.36981 kDa; The average hydrophilicity coefficient is -0.064, which belongs to hydrophilic proteins; The protein instability coefficient is 24.04, which belongs to stable proteins.

```
        10         20         30         40         50         60
MYRFATSLAS KARIARNSTH QIGSRLNWSR NYAAKDIRFG VEARALMLKG VEELADAVRV

        70         80         90        100        110        120
TMGPKGRNVV LEQSFGAPKV TKDGVTVAKS IEFKDRIKNV GASLVKQVAN ATNDVAGDGT

       130        140        150        160        170        180
TCATVLTRAI FTEGCKSVAA GMNAMDLRRG ISMAVDAVVT NLKSRAKMIS TSEEIAQVGT

       190        200        210        220        230        240
ISANGEREIG ELIAKAMEKV GKEGVITISD GKTLYNELEV VEGMKLDRGY ISPYFITNQK

       250        260        270        280        290        300
TQKCELENPL VLIHEKKISN IHAVVKVLEM ALKTQRPLLI VAEDVESEAL ATLILNKLRA

       310        320        330        340        350        360
GIKVCAIKAP GFGENRKAAL QDLATLTGGE LITEELGLNL EKAELDMLGT CKKVTISKDD

       370        380        390        400        410        420
TVILDGAGDK KAIEERCEQI RSAIELSTSD YDKEKLQERL AKLSGGVAVL KIGGASEAEV

       430        440        450        460        470        480
SEKKDRVTDA LNATKAAVEE GIVPGGGVAL LYASKELDKL PTSNFDQKIG VQIIQNALKT

       490        500        510        520        530        540
PVFTIASNAG VEGAVVVGKL LEQDNPDLGY DAAKGEYVDM VKAGIIDPLK VIRTALVDAA

       550        560        570
SVSSLMTTTE AVVVELPKDE KEVPAMGGGM GGMDY
```

Figure 3.3 Amino acid sequence of grape CPN60

3.4 Secondary and tertiary structure analysis of CPN60 protein

Protein secondary structure analysis is an important component of studying proteins. Using online software SOPMA to predict the protein secondary structure of grape CPN60 gene, the analysis results showed that the alpha helix accounted for 55.83% of the secondary structure of grape CPN60 protein, Extended strand accounts for 11.83%, Beta turn accounts for 9.04%, while random coil accounts for 23.30%. It can be inferred that the secondary structure of grape CPN60 protein is mainly composed of irregular curls and alpha helices (Figure 3-4). It can be inferred that the secondary structure of grape CPN60 protein is mainly composed of irregular curls and alpha helices. We used SWISS-MODEL online software to model the protein tertiary structure of grape CPN60 (Figure 3-5), in order to verify the above prediction results of the protein secondary structure of grape CPN60. From the graph, we can see that the protein structure of grape CPN60 is mainly composed of alpha helix

structure and irregular curl structure. The predicted protein secondary structure is consistent with the above results.

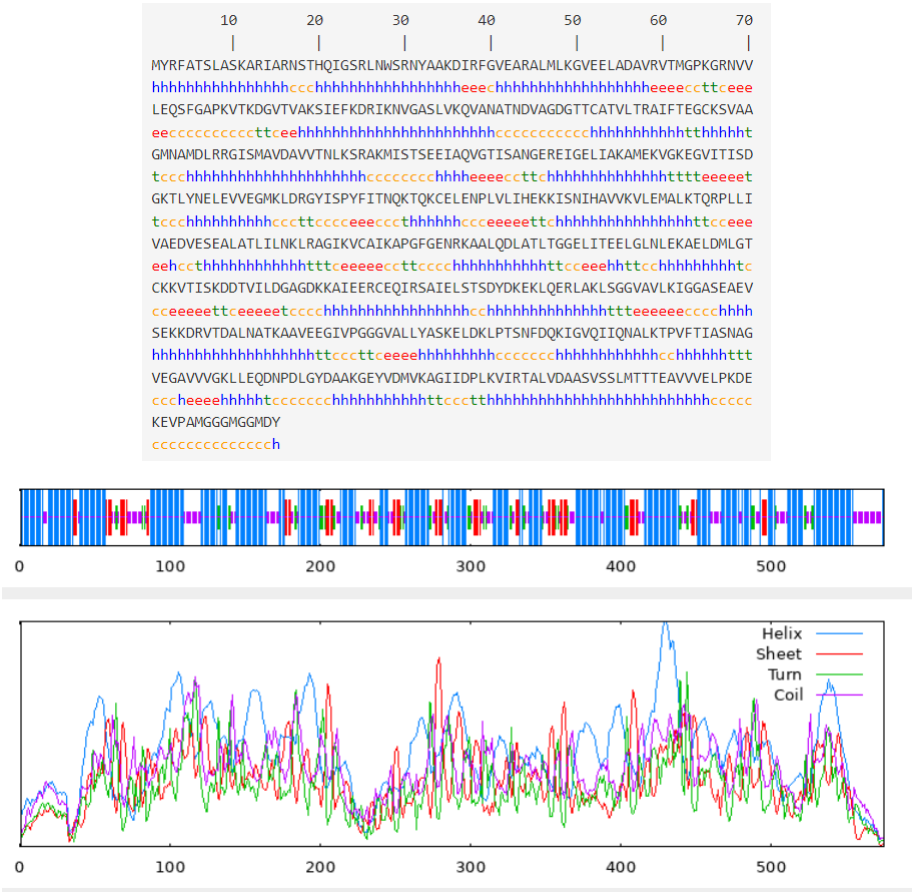


Figure 3-4 Secondary structure analysis of proteins

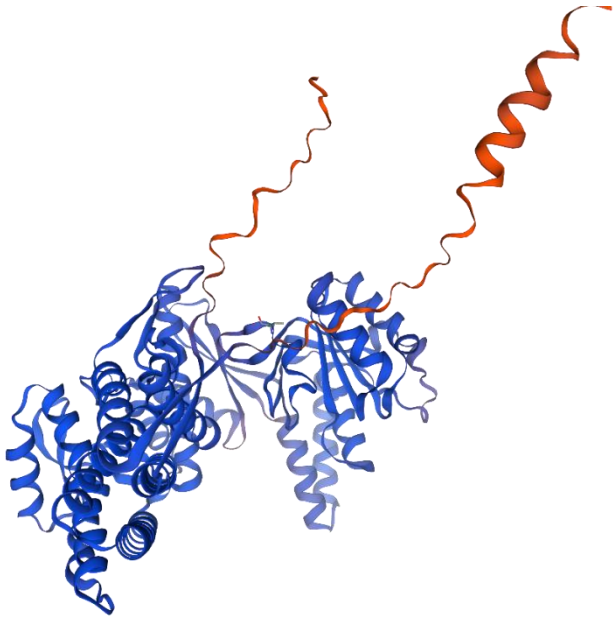


Figure 3-5 Prediction of the Tertiary Structure of CPN60 Protein

3.5 Protein interaction and functional analysis

Using the online software STRING to predict the interaction protein of grape CPN60 (Figure 3-5), it was predicted that it has interaction relationships with multiple proteins, including cpn21, D7SIZ7_VITM, F6GVE3_VITVI, F6HYK6_VITVI, F6HCT7_VITVI, D7T160, F6HN72_VITVI, D7T1A7_VITVI, D7TQS3_VITVI, and VITISV036880. GO biological process analysis shows that CPN60 protein mainly participates in processes such as mitochondrial binding, ATP binding, ATP dependent protein folding chaperone, protein folding, and protein refolding, as shown in Table 1. The results indicate that CPN60 protein is related to mitochondrial protein input and macromolecular assembly, which can promote correct folding of input proteins, prevent erroneous folding, and promote the refolding and correct assembly of unfolded peptides produced in the mitochondrial matrix under stress conditions.

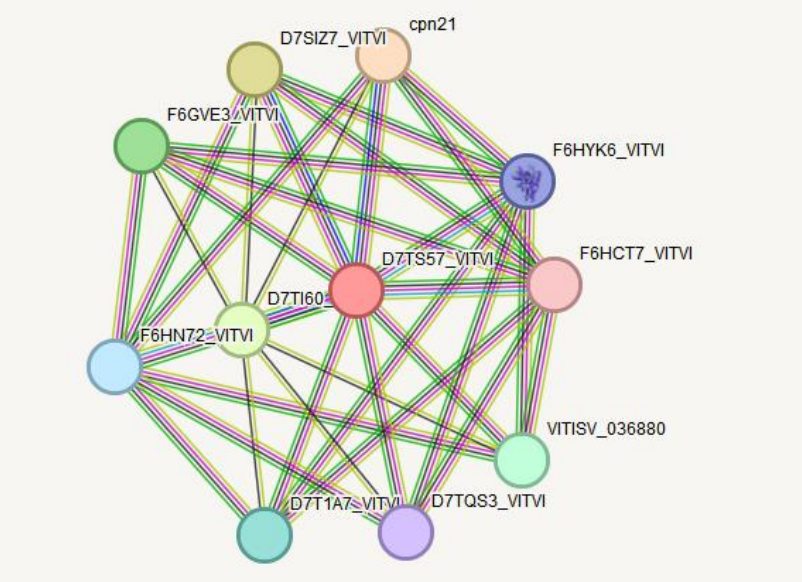


Figure 3-5 CPN60 protein interaction analysis

Table 3-1 **GO Function Annotations**

ASPECT	TERM
Cellular Component	mitochondrion Source:GO_Central 1 Publication
Molecular Function	ATP binding Source:UniProtKB-KW
Molecular Function	ATP-dependent protein folding chaperone Source:InterPro
Biological Process	protein folding Source:GO_Central 1 Publication
Biological Process	protein refolding Source:InterPro

CONCLUSIONS

The multifunctionality of chaperone proteins in photosynthesis reflects their complexity and importance in plant physiological processes, and is a key factor for plants to adapt to environmental changes and maintain efficient photosynthesis. Therefore, genetic engineering can be used to enhance the function of companion proteins, thereby enhancing the photosynthetic efficiency and adaptability to environmental stress of plants. This has potential application value for improving crop yield and improving crop quality. This study cloned the CPN60 gene from eight grape varieties, including 'Creasen seedless',' Jiamei ',' Meile ',' Chardonnay ',' Shiraz ', and grape rootstocks' 1103P', '5BB', and '140R', with a band length of 1728bp. Bioinformatics analysis shows that the CPN60 gene encodes a total of 575 amino acids and is an acidic protein with a molecular weight of 61.36981 kDa; The average hydrophilicity coefficient is -0.064, which belongs to hydrophilic proteins. The protein instability coefficient is 24.04, which belongs to stable proteins. And the secondary and tertiary structures of CPN60 protein were predicted, while protein interactions and functions were analyzed.

The results showed that the structure of grape CPN60 was extremely conservative, with no differences among the 8 varieties, and the overall protein multi-level structure was complete and consistent. It is speculated that the function of this gene is extremely conserved, and its mutation may easily lead to the loss of important functions. There are many interacting proteins encoded by this gene, which may be involved in the regulation of multiple physiological functions.

This study lays the foundation for further exploration of the biological characteristics, molecular mechanisms, and novel genes of grape CPN60 gene.

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