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 **Effects of overexpression of ZmSUS1 gene on drought resistance of maize**

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

Completed: student of group BEBT-20
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Kyiv 2024

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

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Borui Zhang

1. Thesis topic **Effects of overexpression of ZmSUS1 gene on drought resistance of maize**

scientific supervisor Liubov Zelena, PhD
approved by the order of KNUTD "__"_____20__, №____

2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of 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 _____ Borui ZHANG

Scientific supervisor _____Liubov ZELENA

SUMMARY

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Due to climate changes such as water shortage and global warming, crops are increasingly affected by drought stress during their development in today's world, and drought has become one of the main natural factors that reduce crop production.

Sucrose synthase (SUS), as a key enzyme for regulating sucrose decomposition, responds to drought stress by regulating sucrose catabolism and affecting soluble sugar content in plants. In this paper, the phylogenetic tree of *SUS* gene family was constructed to analyze the genetic relationship of *SUS* genes in different plant species, and the cis-acting elements in the promoter of maize *ZmSUS1* gene were analyzed.

In this study, *ZmSUS1* gene was cloned from maize and transgenic maize plants expressing *ZmSUS1* were successfully constructed by Agrobacterium transformation. Molecular identification of transgenic maize lines was carried out by *BAR* gene PCR and qRT-PCR of *ZmSUS1* gene. Simulated drought experiments were carried out on the identified positive lines and CK lines of maize. It was found that compared with CK lines of maize, transgenic maize lines had higher expression of *ZmSUS1* gene, better growth phenotype and stronger drought resistance. The relative water content, ABA and proline contents of transgenic maize lines also increased greatly. In addition, the soluble sugar content in transgenic corn is also higher than that in CK plants. As a result under drought stress, *ZmSUS1* gene can maintain higher water content in plant leaves by regulating sugar metabolism and the steady state of soluble sugar in the body, thus significantly improving the drought resistance of maize. This study provides important insights and technical strategies for further analyzing the mechanism of maize *ZmSUS1* gene responding to drought stress and improving maize drought resistance through molecular breeding technology.

Keywords :ZmSUS1, maize, drought stress, sucrose metabolism.

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INTRODUCTION

In this study, the relationship of SUS genes in different plant species was analyzed by constructing SUS evolutionary tree, and various regulatory elements of the ZmSUS1 gene of transgenic plants and CK plants, phenotype of drought-treated maize and a series of physiological and biochemical indicators, and confirmed that the overexpression of ZmSUS1 gene improved the drought tolerance of transgenic maize. This study provides new ideas and genetic resources for cultivating drought-resistant crops.

The relevance of the topic is *ZmSUS1*; maize; drought stress; sucrose metabolism

The purpose of the study is the Effect of *ZmSUS1* genes on drought resistance in maize.

This study was devoted to the revealing of the mechanism of the *ZmSUS1* gene in drought resistance in maize.

By analysing the cis-acting elements in the promoter region of the *ZmSUS1* gene, its important role in the gene expression process was confirmed.

Transgenic maize lines overexpressing the *ZmSUS1* gene were constructed, and phenotypic analyses and physiological and biochemical indexes were performed in a simulated drought environment to investigate the mechanism and potential of the *ZmSUS1* gene to enhance drought tolerance in maize.

The object of the study is Maize

Research methods is Genetic engineering technology

The scientific novelty is The *ZmSUS1* gene in maize drought resistance is less well studied

The practical significance of the results obtained is *ZmSUS1* Gene can improve the drought resistance in maize

CHAPTER 1
LITERATURE REVIEW

1.1 Effects of drought on plants and adaptation mechanism of plants to drought

1.1.1 Effects of drought on plants. Plants are affected by various stresses such as temperature, water, pests and diseases in the process of growth and development, among which drought is the most common abiotic stress factor affecting plants, which not only causes serious crop yield reduction, but also poses a severe challenge to farmers' life and livelihood [1,2].

Extreme events caused by climate change, such as global warming and El Niñ o phenomenon, have a particularly serious impact on agriculture in China. From 2008 to 2018, the agricultural losses in China due to disasters totaled 976 billion yuan, of which the losses caused by drought were the most serious [3]. It is predicted that by 2030, seasonal drought will bring more severe challenges to crop growth.

Due to climate change and temperature rise, crop transpiration rate is accelerated and water evaporation is enhanced, which leads to crop growth retardation and even death. In addition, high temperature also affects the photosynthesis of crops, thus affecting the absorption of nutrients and the transformation of sugar, shortening the growth cycle of crops and affecting crop yield.

1.1.2 Adaptation and response of plants under drought stress. In order to cope with drought stress, plants in arid areas have undergone adaptive variation, such as changes in various molecular, biochemical and physiological mechanisms. In addition, many genes responding to drought stress have been found in plants, such as some enzymes, transcription factors, signal molecules and regulatory proteins that encode or regulate the metabolism of carbohydrates, lipids, phenylpropanes and plant hormones (such as ABA) [4–9].

(1) Macro-control of plants in response to drought

When plants respond to drought stress, there are three main coping mechanisms:

Escape, plants respond to drought by adjusting growth cycle, slowing growth rate, closing stomata, and adjusting roots to reduce water evaporation. Some plants mitigate the effects of drought by completing key life cycles around the drought period. Drought escape is also reflected in the rapid response of plants to water stress, such as leaf folding and wilting to reduce water loss in some tissues [10].

Avoidance means that plants such as sorghum and wheat can avoid drought stress by reducing water loss and increasing water under drought conditions, including reducing transpiration, reducing stomatal conductance, reducing leaf area, increasing root depth and surface area, and increasing water absorption capacity [11]. So as to avoid the influence of drought stress, so that plants can still maintain a high water state in arid environment [12].

Drought Tolerance, desert plants such as cactus and seabuckthorn can maintain cell osmotic pressure, protect cell membrane and protein, reduce damage caused by drought, regulate stomatal movement and photosynthesis, and maintain physiological functions by accumulating osmotic pressure regulating substances such as proline, glycine and betaine in low water potential environment.

(2) Micro-control of plants in response to drought

Plants adapt to the water-deficient environment through a series of molecular mechanisms under drought conditions.

1) Plant hormones play a key role in responding to drought stress, which can promote seed dormancy, inhibit germination and reduce transpiration of leaves by regulating various physiological processes, such as stomatal closure, root regulation, influence on soil microbial community, transcription and activation of gene expression after transcription, and metabolic changes, thus regulating plant resistance to drought [13].

Abscisic Acid(ABA) is a plant hormone with chemical formula of C15H20O4, and its molecular structure contains a cyclic group and three side chains. The side chain includes a nine-carbon fatty acid and a three-carbon sugar alcohol. This

structural feature endows ABA with acidity and lipophilicity, which makes it play a role in plant synthesis, metabolism, growth and development and stress response.

ABA is an organic acid with high solubility in organic solvents. Because it contains long-chain fatty acid side chains, it has high lipophilicity and good thermal stability. ABA binds to cell surface receptors, activates protein phosphatase 2C(PP2C) and SnRK2/SnRK3 protein kinases, and then regulates the activity of a series of transcription factors, which then enter the nucleus and regulate the expression of related genes.

ABA can activate plant stress response, close stomata, adjust root environment, activate transcription and post-transcriptional gene expression. These processes work together to help plants improve their ability to resist stress. Under drought stress, the ABA level in leaves increased, which led to the opening of K+ outflow channel on guard cell membrane, increased K+ outflow, inhibited the activity of K+ inflow channel, and reduced K+ inflow, which eventually led to the reduction or closure of leaf stomata opening, reduced water evaporation, and enhanced water retention and drought tolerance of plants.

ABA can prevent membrane lipid from being oxidized by regulating the activities of various enzymes in plants, and protect the structural integrity of membrane, so that it can better adapt to adversity stress [14]. In addition, ABA can reduce the water consumed by metabolism and improve the water use efficiency by inhibiting plant growth and changing the ratio between rhizomes [15].

2) Transcription factor is a molecular switch for regulating gene expression, which can activate or inhibit the gene expression by binding to a specific gene promoter. Some transcription factors, such as DREB/CBF, NAC, MYB and ERF, have been proved to regulate the expression of drought-responsive genes, thus affecting the growth cycle and water use of plants. Plants can improve photosynthetic efficiency by regulating the expression of photosynthesis-related genes and complete their life cycle quickly. For example, regulating the activity of key enzymes in photosynthesis, such as Rubisco, can help plants to fix carbon dioxide more

effectively and quickly under drought conditions, thus increasing the speed of photosynthesis[16].

3) Plant Aquaporins, AQPs play an important role in water transport. Under drought conditions, plants can regulate the expression of AQPs to control the permeability of cell membrane to water, thus reducing water loss [17].

4) Organic osmotic regulators are plants that accumulate organic substances to increase the concentration of cell sap, reduce osmotic potential and improve the water absorption or retention capacity of cells under adversity (drought, low temperature, high temperature, salinity, etc.). In plants, osmotic adjustment substances mainly include inorganic ions and organic solutes. Inorganic ions such as Na+, Cl-, etc., while organic solutes include proline, betaine, glycerol, etc.

Proline is a cyclic imino acid with a chemical formula of C5H9NO2 and a molecular weight of 115.13 [18]. Once the peptide chain enters, it can be hydroxylated to form 4- hydroxyproline, which exists in many plant protein and plays a key role in cell wall formation [19].

Under adversity stress (such as drought, high temperature and low temperature), plants reduce osmotic pressure by increasing the content of organic osmotic regulators such as proline, and participate in scavenging free radicals and reducing cell acidity, thus enhancing stress resistance [20]. Proline is also used as an anti-dehydration agent, which can reduce ammonia toxicity caused by protein hydrolysis, store nitrogen and carbon, and provide respiratory matrix and carbon source for recovery after adversity is relieved[21].

1.2 Sugar metabolism and abiotic stress

Plant sugar metabolism needs to go through a series of complex biochemical reactions, including the formation, decomposition and transformation of sugar, which is very important for plant growth, development, productivity and cycle. Plants produce glucose through photosynthesis, which is then converted into other sugars such as sugar, sucrose and starch by enzymes. These soluble sugars are the key

substrates for plant growth and carbon metabolism. The produced sugar participates in cell metabolism through respiration, producing energy and ATP.

Soluble sugar in plants is mainly composed of glucose, sucrose and fructose, which is one of the important indexes reflecting plant growth status and environmental adaptability. Soluble sugar content will change with environmental stress (such as drought, salt damage and low temperature) to provide energy, regulate cell osmotic pressure and protect cell structure.

Sugar metabolism can regulate the growth and development of plants, especially in the stage of seed germination and early seedling growth. Soluble sugar can inhibit the expression of α -amylase and other genes, thus inhibiting seed germination. Gene expression is regulated through signal transduction pathways, such as protein phosphorylation and dephosphorylation, calcium signal, etc., and the activities of enzymes related to glucose metabolism are regulated.

1.3 sucrose metabolism and key enzymes

1.3.1 The role of sucrose. In the process of photosynthesis in higher plants, the produced glucose is usually converted into sucrose, which is an important form of storing energy and carbon source, and is used for plant growth and development, such as promoting the growth and development of roots, pollen vitality and pollen tube elongation, fruit development and starch accumulation [22].

Sucrose plays an important role in plants' resistance to abiotic stresses (including environmental stresses such as drought, salt damage, low temperature, high temperature, too strong or too weak light) [23]. Sucrose is a osmotic adjustment substance. In arid environment, plants can maintain the normal osmotic balance of cells and keep water to reduce the damage of stress to cell membrane and protein [24]. Plants need to consume a lot of energy under stress. As an energy storage material, sucrose produces ATP through glycolysis, which provides energy for plants, maintains physiological functions and promotes the repair of damaged cells. As a signal molecule, sucrose regulates plant gene expression, promotes the synthesis of stress proteins, and enhances tolerance to adapt to stress. In addition, sucrose affects

the activity or stability of transcription factors, activates or inhibits intracellular signal transduction pathways (such as MAPK and Ca2+ signals), thus regulating the synthesis and transmission of plant hormones. Under drought stress, plants will produce a large number of reactive oxygen species (ROS), which will damage the cell structure. Sucrose provides carbon source and energy, promotes the synthesis of antioxidant enzymes, helps plants remove ROS and reduces oxidative stress; Sucrose is involved in regulating the synthesis and reconstruction of cell wall, enhancing the strength of cell wall to resist the pressure of external environment. This strengthening of cell wall helps plant cells to maintain the stability of morphology and structure under drought stress.

1.3.2 Sucrose synthesis and decomposition and its key enzymes. Plant sucrose metabolism mainly includes two processes: synthesis and decomposition. Sucrose is mainly synthesized in leaves, using UDP- glucose as the substrate, catalyzed by sucrose phosphate synthase (SPS) and sucrose phosphatase (SPP) [25].

(1) Sucrose phosphate synthaseSucrose phosphate synthase (SPS) is the key rate-limiting enzyme in sucrose biosynthesis pathway, which catalyzes the synthesis of sucrose -6- phosphate, and then it is dephosphorized by sucrose phosphatase (SPP) to produce sucrose [26]. SPS plays a key role in plant growth, development and environmental stress response, regulating carbohydrate distribution, affecting growth and fruit ripening, and participating in starch and cellulose synthesis. As a "reserved" glycosyltransferase, SPS maintains the configuration of hetero-carbon of sugar molecules during the catalytic process. The key amino acid residues in its catalytic center, such as histidine and glutamic acid, participate in the formation of hydrogen bond network and stabilize intermediate products.

(2) Sucrose phosphatase (SPP)The last step of sucrose synthesis is catalyzed by SPP, which contains about 500 amino acids and has a molecular weight of 50−60 kD. SPP hydrolyzes sucrose phosphate (sucrose -6- phosphate) into sucrose and inorganic phosphate. This process takes place in the cytoplasm and the reaction is irreversible. It is found that SPP is involved in regulating the transport and accumulation of photosynthetic products in plants, regulating the sugar metabolism and distribution in

the middle and late stages of fruit, thus affecting the growth and development of fruit, and also plays a role in the process of plant stress resistance.

(3) Sucrose invertase (INV)When plants respond to drought stress, sucrose catabolism is very important, which is mainly catalyzed by sucrose synthase (SUS) and sucrose invertase (INV). Sucrose invertase (INV) is a member of glycosyl hydrolase family, which consists of two domains: a sucrose binding domain and an active center that catalyzes hydrolysis reaction. There are some important amino acid residues in the active center, which play an important role in substrate hydrolysis.

The molecular weight of sucrose invertase varies among different species and types, usually tens to thousands of kDa. According to its optimum pH value and distribution, sucrose invertase can be divided into two categories: acid invertase and alkaline/neutral invertase) [27] .

Sucrose invertase (INV) irreversibly catalyzes the hydrolysis of sucrose to glucose and fructose. In the process of catalysis, the amino acid residues in the active center of the enzyme form a temporary complex with sucrose molecules, and then sucrose is decomposed by hydrolysis reaction. It mainly INVolves acid-base catalysis, in which the amino acid residue of inv active center participates in the reaction as acid or base. Invi plays an important physiological role in plants, and its distribution and activity are strictly regulated to adapt to different physiological needs and external environmental conditions.

Sucrose invertase includes three types: cell wall, cytoplasm and vacuole invertase. Cell wall invertase is involved in sucrose unloading and the growth and development of sink tissue, which plays an important role in the development of seeds and fruits. Cytoplasmic invertase is involved in plant root and reproductive development [27]. Vacuolar invertase is involved in cell osmotic adjustment and sugar accumulation in fruits and tubers [28].

Sucrose invertase plays an important role in regulating plants' response to abiotic stress, such as drought and high temperature, especially in the consumption of hexose. In the process of interaction between plants and pathogens, cell wall

invertase plays an important role in the process of pathogen obtaining host sugar, and activates host defense response.

1.4 Research progress of sucrose synthase

1.4.1 Structure and Activity of Sucrose Synthase (SUS). Sucrose synthase (SUS) is a glycosyltransferase with a molecular weight of about 100-150 kDa. It exists in the form of tetramer or dimer, and tetramer is considered as an active form [29]. Each subunit contains a UDP binding site and a glycosyltransferase site, and these sites interact through flexible structures, so that the enzyme can adjust its conformation in the catalytic process. The structural characteristics of SUS enable it to transfer sugar groups between two active sites, thus catalyzing the synthesis or decomposition of sucrose. Specifically, SUS synthesizes sucrose by transferring glucose groups from UDP- glucose to fructose, which occurs in the active center of glycosyltransferase site. In addition, UDP binding sites play a key role in maintining the stability and catalytic activity of the enzyme [30]**.**

The activity of SUS is regulated by many factors, including environmental factors (light, temperature and moisture) and chemical factors (phosphorylation, carbohydrates and hormones, etc.). Phosphorylation is the key mechanism of SUS activity regulation, and the catalytic activity of the enzyme is regulated by changing the conformation of the enzyme. In addition, soluble sugars such as glucose, fructose and sucrose can also regulate the stability and catalytic activity of SUS through the interaction with enzymes.

In plant cells, the distribution of SUS is tissue-specific, mainly distributed in cytoplasm, plasma membrane and cell wall. The expression and activity in source tissues such as leaves are different from those in sink tissues such as seeds, roots and tubers. This specific expression and activity regulation is helpful for carbon distribution and utilization of plants in different growth stages and environments.

1.4.2 Functions of SUS. Sucrose synthase plays an important role in plant growth and development. SUS with high activity converts photosynthetic products into sucrose and transports it to seeds for storage. Under environmental stress, SUS regulates sucrose content to help plants cope with adversity. In addition, SUS is related to the synthesis of plant cell walls, and UDP- glucose is provided as a substrate for cellulose synthesis. Therefore, SUS shows versatility in plant growth and development and in response to environmental changes.

The first cloned and sequenced SUS gene originated from Shrinkken(Sh1) gene of maize [31]. Through the study of SUS, it is found that there are significant differences in the types and quantities of SUS genes in different plants. For example, six SUS genes were found in Arabidopsis, rice and tomato, while three SUS genes, namely Sh1, SUS1 and SUS3 (sometimes called SUS2), were confirmed in maize [32].

Because of its important role in plant growth and response to adversity, SUS has become a potential target for genetic engineering and crop improvement. By controlling the expression of SUS, scientists try to improve the yield, quality and tolerance to environmental stress of crops. In recent years, many studies have shown that SUS in plants is involved in many metabolic processes, including the transport and distribution of sucrose, the synthesis of starch and cellulose, the composition of cell walls, and coping with biotic and abiotic stresses. Among them, most studies focused on the effects of SUS on starch synthesis in fruits and grain development, such as starch synthesis in maize endosperm and rice grains [33,34]. However, recent studies have shown that SUS does not participate in the synthesis of starch and cellulose in Arabidopsis leaves [35]. In addition, the research on plant stress resistance of SUS mainly focuses on hypoxia and low temperature stress, but there is relatively little research on plant drought resistance.

1.5 The purpose and significance of this study

1.5.1 Purpose and task. Corn is one of the most widely planted crops in the world. It is not only a key source of food and feed, but also widely used in industrial

production. Its annual output ranks first among crops and has extremely high economic value. However, due to the sensitivity of maize to drought, drought stress has become a key abiotic stress factor affecting its normal growth and yield. The purpose of this study is to reveal the mechanism of *ZmSUS1* gene in maize drought resistance. Through the analysis of cis-acting elements in the promoter region of *ZmSUS1* gene, the important role of cis-acting elements in gene expression was confirmed. The mechanism and potential of *ZmSUS1* gene in improving drought resistance of maize were explored by constructing transgenic maize lines expressing *ZmSUS1* gene, and carrying out phenotypic analysis and determination of various physiological and biochemical indexes under simulated drought environment.

1.5.2 Scientific significance and application value. Compared with mutation breeding, genetic engineering is a more direct and accurate method of gene mutation. Precise operations, such as insertion, knockout or substitution, are performed in the genome to achieve specific genetic mutations, such as drought resistance, insect resistance, disease resistance and salt tolerance.

In recent years, global warming and extreme weather events have become more frequent, and drought has become a key abiotic stress factor affecting crop growth and yield. When dealing with extreme environments such as drought, genetic engineering technology provides a new way to solve this problem. By studying the response mechanism of plants to drought stress, many genes with drought resistance were found. These genes are introduced into crops through genetic engineering to enhance their drought resistance. The new drought-resistant varieties cultivated by genetic engineering technology can not only adapt to the arid environment, but also maintain normal growth and increase yield, which provides an important guarantee for the sustainable development of agriculture. Therefore, the application of genetic engineering technology in cultivating drought-resistant varieties has great potential and significance, and provides a new solution to cope with the increasingly severe drought environment.

In this study, transgenic maize lines with over-expression of *ZmSUS1* gene were constructed by genetic engineering technology. It was found that over-

expression of *ZmSUS1* improved the drought resistance of maize and clarified the key role of *ZmSUS1* gene in drought resistance, which provided an important reference for improving the drought resistance of crops by genetic engineering technology

1.5.3 Research ideas. In this study, the phylogenetic tree of SUS was constructed to analyze the genetic relationship of SUS genes in different plant species, and various regulatory elements of *ZmSUS1* promoter sequence were analyzed to preliminarily understand the functions and functions of these regulatory elements. In this study, the gene *ZmSUS1* was overexpressed in maize by genetic engineering technology. The transgenic plants and CK plants were treated by hydroponics to simulate drought, and the phenotypic analysis and a series of physiological and biochemical indexes of drought-treated maize seedlings were tested. It was confirmed that the overexpression of *ZmSUS1* gene improved the drought resistance of transgenic maize. This study provides new ideas and genetic resources for cultivating drought-resistant crops by genetic engineering**.**

CHAPTER 2
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Experimental materials and apparatus

2.1.1 Plant materials. The maize inbred line used in this study was KN5585 from the Plant Microbial Biotechnology Laboratory of Qilu University of Technology.

2.1.2 Main reagents. PrimeScript RT reagent Kit with gDNA Eraser Reverse Transcription Kit, MiniBEST Universal RNA Extraction Kit Plant RNA Extraction Kit, SYBR Green RT-PCR Kit (TaKaRa); Phanta Max Super-Fidelity DNA Polymerase (Novozymes, Nanjing, China); Plant Abscisic Acid (ABA) Enzyme Immunoassay Kit (Jingmei, Jiangsu, China); Standard ninhydrin, proline, fructose, glucose, sucrose (Sigma**).**

2.1.3 Main instruments and equipment

Table 2.1 **Main experimental instruments and manufacturers**

Instrument	Model	Manufacturer
PCR Instruments	C1000 Touch	BIO-RID
real-time PCR instrument	MJ3760D	Thermo Fisher
Small vertical electrophoresis tank	Mini-PROTEANTetra	BIO-RID
Refrigerator (4℃, -20℃)	BCD-539WT	Haier
High-speed freezing centrifuge	Centrifuge 5424R	Eppendorf
Ultra-high speed centrifuge	Centrifuge 5840R	Eppendorf

2.1.4 Primers

Table 2.2 **Primer sequences used in this chapter**

Gene ID	Forward primer (5' to 3')	Reverse primer (5' to 3')
bar（PCR）	ATGAGCCCAGAACGACGCC	TCAGATCTCGGTGACGGGC
ZmActin（RT-PCR）	ATCACCATTGGGTCAGAAAGG	GTGCTGAGAGAAGCCAAAATAGAG
ZmSUS1（RT-PCR）	AGCAGTACAACCTGAACGGG	TGTCGAAGAAGTCCACGAGC

2.2 SUS phylogenetic tree construction and ZmSUS1 gene promoter analysis

2.2.1 SUS multiple sequence comparison and phylogenetic tree construction. The MEGA11.0 software was used to construct the evolutionary tree. Firstly, the amino acid sequences of different plants in the SUS gene family, including maize, rice, Arabidopsis and other species, involving a total of 28 amino acid sequences, were downloaded via NBCI (https://www.ncbi.nlm.nih.gov/), and the acquired sequences were formatted, and the amino acid sequences were aligned by codon selection using the ClustalW algorithm and subjected to multiple sequence comparison. The most suitable substitution model to describe the probability of amino acid or nucleotide changes over time was then selected using the model selection tool of MEGA11.0.

Evolutionary trees were finally constructed using the maximum likelihood (ML) method of the MEGA11.0 software, with default options selected for the other data, and evolutionary tree analyses were performed using Bootstrap and 1000 cycles. Phyl ogenetic evolutionary trees were landscaped using the online website evolview (https: //www.evolgenius.info/evolview)[36].

2.2.2 Promoter cis-acting element analysis of the *ZmSUS1* gene. In NBCI, the promoter sequence 2.0kb upstream of the *ZmSUS1* gene in reference genome B73 was selected for promoter cis-acting regulatory element analysis using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html).

2.3 Molecular validation of trans-ZmSUS1 gene maize

2.3.1 Acquisition of trans-*ZmSUS1* gene maize. The Zm00001d047253 gene was cloned from maize, ligated to EYFP and inserted into a plant expression vector containing the 35S promoter and *BAR* gene. The recombinant vector was introduced into Agrobacterium EHA105 strain using cold-excitation method, and then cultured in medium containing bis(propylammonium)phosphine for a period of time to verify whether the transformation was successful. Agrobacterium was introduced into the endosperm of maize, and the genetic transformation included the steps of stripping embryo infestation, co-cultivation, healing induction, differentiation into seedlings, and induction of rooting. Eventually, positively transformed seedlings were obtained and planted in the greenhouse of Qilu University of Technology. The work was done by the laboratory in advance.

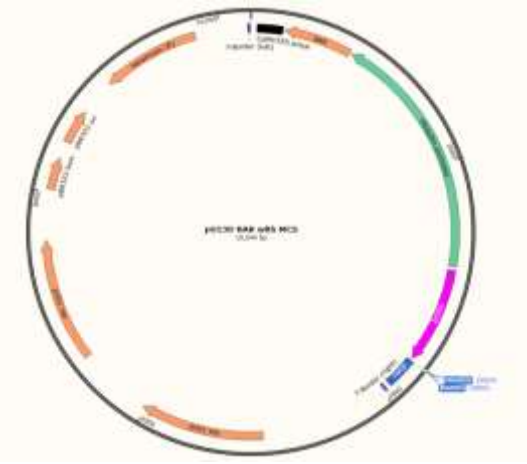


Figure 2.1 – Schematic diagram of the *PU 130-BAR* vector

2.3.2 Drought stress treatment and phenotypic observation. PEG solution is a high osmotic pressure solution and treating plants so that the roots are unable to absorb water can simulate drought stress. Drought stress was simulated using 18% PEG 6000 when maize was grown to the three-leaf stage. After treatment, samples were taken at 0, 2, 6, 12, 24, 48 and 72 hours to compare maize leaves under normal conditions and PEG treatment. The samples were treated in liquid nitrogen and stored in a -80C refrigerator. Meanwhile, the nutrient solution was changed every two days to ensure timely replenishment of fresh nutrient solution.

The maize phenotypes were observed at 18% PEG6000 treatment, three days a nd seven days after treatment, respectively.

2.3.3 PCR detection of transgenic maize. In this study, we used M5 FTL MIX kit without extracting genomic DNA and directly designed primers with herbicide *Bar* gene sequence for PCR verification. About 0.1g of plant leaves were taken, 40μl of lysate was added, ground and boiled for 10min, and the supernatant was centrifuged to be used as PCR template.The PCR reaction system was 20μL, including 1μL each of F/R primers, 1μL of the target gene template, 10μL of PCR mix, and 7μL of ddH2O.Amplification conditions were as follows: pre-denaturation at 95℃ for 3min, 30 cycles (94℃ 25s, 58℃ 25s, 72℃ 30s), and finally 72℃ extension for 5min. PCR products were detected by agarose gel electrophoresis.

2.3.4 Real-Time PCR assay of transgenic maize

(1) Plant total RNA extraction

Total RNA was isolated from maize leaves using the TaKaRa MiniBEST Universal RNA Extraction Kit (TAKARA) kit.The steps were as follows:

During maize leaf RNA extraction, fresh or ultra-low-temperature preserved plant tissue samples were rapidly transferred to a pre-cooled liquid nitrogen mortar and ground to powder while adding liquid nitrogen to ensure no particles to safeguard RNA quality and yield. The ground sample was transferred to a 1.5 ml sterilised tube containing Lysis Buffer RL (to which 50× DTT Solution had been added) and blown with a pipette to eliminate precipitation. If the lysate is viscous, repeat the blowing 5-10 times. Centrifuge the lysate at 12,000 rpm for 5 minutes at 4° C and then

carefully transfer the supernatant to a new 1.5 ml RNase Free Tube. Mount the gDNA Eraser Spin Column on a 2 ml Collection Tube, transfer the supernatant to the Column and centrifuge for 1 minute. Discard the gDNA Eraser Spin Column and retain the filtrate. Add an equal volume of anhydrous ethanol to the primary purified RNA filtrate, mix well and transfer to the RNA Spin Column, centrifuge for 1 min and discard the filtrate. Add 500 μl of Buffer RWA to the RNA Spin Column, centrifuge for 30 seconds and discard the filtrate. Add 600 μl of Buffer RWB, centrifuge for 30 seconds and discard the filtrate. Add Buffer RWB along the wall of the RNA Spin Column tube to fully clean the salt from the tube wall.

If the genomic DNA content in the tissue is high or there are special requirements for RNA purity, DNaseI treatment can be performed on the RNA Spin Column membrane. Prepare DNase I reaction solution and add it to the centre of the RNA Spin Column membrane, let it stand for 15 minutes at room temperature and then add Buffer RWB, centrifuge for 30 seconds and discard the filtrate. Repeat the addition of 600 μl Buffer RWB to the RNA Spin Column, then centrifuge at 12,000 rpm for 30 s and discard the filtrate. Reposition the RNA Spin Column on a 2 ml Collection Tube and centrifuge for 2 minutes. Place the RNA Spin Column into a 1.5 ml RNase Free Collection Tube, add 50-200 μl RNase Free dH2O or 0.1% DEPC treated water, leave at room temperature for 5 minutes and centrifuge for 2 minutes to elute the RNA.

(2) RNA Reverse Transcription

Prepare the reaction mixture on ice as follows. To ensure accuracy, prepare more than twice the desired number of reactions of Master Mix, then dispense it into each reaction tube and finally add the RNA sample.

Reagent	Application amount
5×gDNA Eraser Buffer	2.0 μl
gDNA Eraser	1.0 μl
Total RNA	2.0 μl

RNase Free dH2O	10.0 μl

↓

42℃2 min

4℃

Reverse transcription was performed using the TaKaRa Reverse Transcription Kit. Prepare the reaction solution on ice. To ensure accuracy, prepare the Master Mix in the desired number of reactions + 2, followed by dispensing 10 μl into each reaction tube. Start the reverse transcription reaction immediately after gentle mixing.

Reagent	Application amount	
Reaction solution for step 1	10.0 μl	
PrimeScript RT Enzyme Mix I	1.0 μl	Master Mix
RT Primer Mix *4	1.0 μl	
5×PrimeScript Buffer 2 （for Real Time）	4.0 μl	
RNase Free dH2O	4.0 μl	
Total	20μl *5	

2.5 Determination of ABA, proline, soluble sugar and sucrose synthase content

Proline content was determined by ninhydrin colourimetric method. During ext raction of plant samples with sulphosalicylic acid, proline is freed into solution. After treatment with acid ninhydrin and heating, the solution turns red, followed by extract ion using toluene, the pigment is transferred to toluene and its colour shade reflects th e proline content. Colorimetric analysis was performed at 520 nm and the proline con tent was calculated from the standard curve or regression equation [37].

In this study, an ELISA kit (Jiangsu Jingmei Biotechnology Co., Ltd., Jiangsu, China) was used to determine the ABA content, which used a double antibody sandw ich method to detect the level of phytobetaine (ABA) in the samples. A solid phase a

ntibody was first made by wrapping purified ABA antibody with a microtiter plate. T hen, ABA antigen is added into the microtiter wells wrapped with monoclonal antibo dy, which binds to HRP-labelled ABA antibody to form an antibody-antigen-enzyme-labelled antibody complex, i.e. ABA antigen. After washing, TMB is added to develo p the colour. TMB first turns blue under the action of HRP enzyme, and then turns ye llow under acidic conditions, which is proportional to the ABA content in the sample, showing a change of shades of colour. Finally, the absorbance (OD value) was meas ured at 450 nm using an enzyme marker and the concentration of ABA in the sample was calculated from the standard curve.

Soluble sugar content was determined by high performance liquid chromatogra phy (HPLC). 0.5 g of corn leaves were weighed and ground in liquid nitrogen, 5 mL of 80% chromatographic ethanol was added and extracted by sonication at 35°C for 3 0 min. centrifugation was performed at 12,000 rpm for 15 min, the supernatant was di luted to 10 mL and rotary evaporated to 2 mL at 60°C, and finally re-dissolved in a 1: 1 mixture of ultrapure water and acetonitrile. The soluble sugar content was measured using a Shimadu LC-10AD detector and an HPX-87H (Bio-Rad) column with 24.8% ultrapure water, 0.2% triethylamine and 75% acetonitrile as mobile phases at a flow r ate of 0.8 mL/min, a detection wavelength of 254 nm, and a column temperature of 4 0°C. The results showed that the soluble sugar content of the leaves were not only me asured by the detection of the soluble sugar content, but also by the analysis of the sol uble sugar content.

2.6 Determination of leaf dry weight, fresh weight and relative water content

The veins and leaf tips of the plants were excised and leaf fresh weight (FW) w as recorded immediately and the leaves were dried in an oven at 85°C and weighed to obtain the dry weight (DW) [38]. For the determination of relative water content (R WC), fresh leaves were quickly weighed to obtain the fresh weight (FW), then the lea ves were immersed in distilled water for 24 h to obtain the saturated fresh weight (T W), and then the leaves were dried in an oven at 85°C to measure the dry weight (D

W), and the RWC was calculated as follows: rwc (%) = 100% × (FW - DW)/(TW -D W) [39]

RESULTS AND DISCUSSION

3.1 *SUS* phylogenetic tree construction

The *SUS* genes of different plants obtained from NCBI are as follows:

Maize (*Zea mays*): *ZmSUS-sh*(CAA26247.1), *ZmSUS1*(AAA33514.1), *ZmSUS 3*(AAM89473.1);

Rice (*Oryza sativa*): *OsSUS1*(CAA46017.1), *OsSUS2*(CAA41774.1), *OsSUS3*(AAC41682.1),*OsSUS4*(BAG95417.1), *OsSUS5*(CAE03896.2), *OsSUS6*(BAD23005. 1) ;

Murphy(*Solanum tuberosum*): *StSUS1*(AAO67719.1), *StSUS2*(AAO34668.1), *StSUS3*(AAA97572.1), *StSUS4*(CAD61188.1);

Barley (*Hordeum vulgare*): *HvSUS1*(CAA46701.1), *HvSUS2*(CAA49551.1);

Sorghum（*Sorghum bicolor*）:*SbSUS1~4* (XM002465116、FJ513325、XM002453052、XM002465258)

Wheat (*Triticum aestivum*): *TaSUS1*(CAA04543.1), *TaSUS2*(CAA03935.1),

Arabidopsis thaliana: *AtSUS1*(NP_197583.1), *AtSUS2*(NP_199730.1), *AtSUS3* (NP_192137.1), *AtSUS4*(NP_566865.2), *AtSUS5*(NP_198534.2), *AtSUS6*(NP_17748 0.1)

the *ZmSUS1* gene is closest in affinity to the sorghum *Sbsus2*, rice *OsSUS1* and wheat *TaSUS1* genes.(Fig. 3.1)

Figure 3.1 – The evolutionary tree of the *SUS* gene family

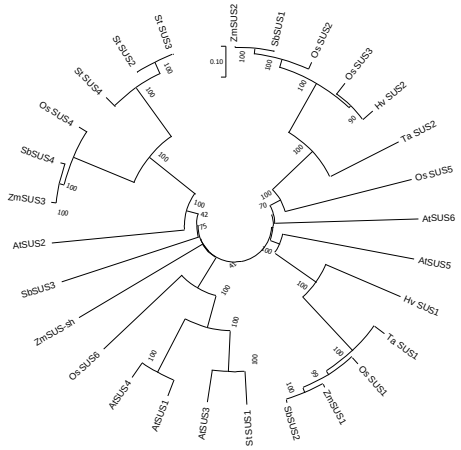
3.2 Sequence analysis of ZmSUS1 promoter

Sequence analysis of the promoter of *ZmSUS1* using Plantcare revealed that its promoter fragment contains regulatory elements such as DREB, STRE , MBS fragment (a binding site for MYB, which is involved in drought stress), CGTCA-motif, and ABA response element (ABRE).

DREB (Dehydration Responsive Element Binding) is a transcription factor found in plants that is mainly involved in response and adaptation to abiotic stresses such as drought, high salt and low temperature. It regulates the expression of a series of adversity-responsive genes through specific binding to DRE (Dehydration Responsive Element), thereby enhancing plant resistance to environmental stresses [40].

STRE (Stress Response Element) is a DNA sequence in a promoter region that responds to environmental stress (e.g., high temperature, acidic conditions, oxidative stress, etc.) and regulates the expression of related genes.

The MYB family of transcription factors consists of proteins containing the MYB structural domain, which consists of 51 to 52 amino acids and contains several conserved amino acid residues and specific spacer sequences. These transcription factors are widespread in eukaryotes and play key roles in several physiological processes such as plant growth, development, metabolism and signalling. They all



contain a conserved MYB-DNA-binding structural domain consisting of one to four

incomplete repeats (R), each encoding three α-helices containing approximately 50 to 53 amino acids.MYB transcription factors insert into the large groove of DNA through their HTH (helix-turn-helix) structure, bind to DNA, and regulate the expression of related genes[41].

Under abiotic stress conditions (e.g., drought, UV, cold stress, high temperature stress, salt stress, etc.), the expression of MYB transcription factors undergoes specific changes and is involved in plant adaptation to the environment. Under drought conditions, MYB transcription factors regulate drought tolerance by modulating the expression of biosynthetic genes, such as flavonoids, waxes, and keratin, and are involved in ABA signalling, which affects stomatal movement and plant drought tolerance.

CGTCA-motif (CGTCA sequence motif) is an important cis-acting element in the regulation of plant gene expression and is involved in plant response to a variety of abiotic stresses (e.g., drought, salinity, low temperature, etc.).

ABRE (Abscisic Acid Responsive Elements) is a DNA sequence in the plant genome, which is recognised and bound by abscisic acid (ABA), and is involved in ABA-regulated gene expression.AREB/ABF (ABA Responsive Element Binding protein/ABRE Binding Factors) is an important cis-acting element in the regulation of plant gene expression. protein/ABRE Binding Factors) transcription factors belong to the basic leucine zip (bZIP) class of proteins, which recognise and regulate the expression of ABA-responsive genes to enhance plant adaptation to environmental stress. These transcription factors play a key role in plant response to drought stress, and can activate downstream gene expression in response to stress signals by binding to their specific and conserved cis-acting elements.

3.3 Expression vector construction, transgenesis and transgene verification

PU130-BAR- ZmSUS1-EYFP expression vector was constructed. Transform maize inbred line KN5585 using Agrobacterium transformation method.(Fig. 3.2)

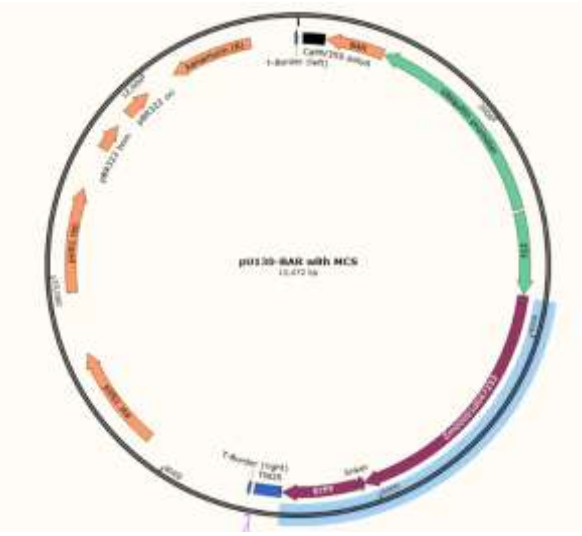


Figure 3.2 – Recombinant vector plasmid

3.3.1 PCR detection of transgenic lines

After a rigorous self-crossing process, pure transgenic maize (L1, L2 and L3) was successfully bred from the T1 generation of transgenic maize. Subsequently, PCR detection of the *BAR* gene was carried out on the pure transgenic maize and the control group, through which, as shown in Figure 3-3, the *Bar* gene was detected in only three transgenic maize strains L1, L2 and L3. It was confirmed that the recombinant vector had been successfully transformed into maize and the trans *ZmSUS1* gene maize plants were successfully constructed.(Fig. 3.3)

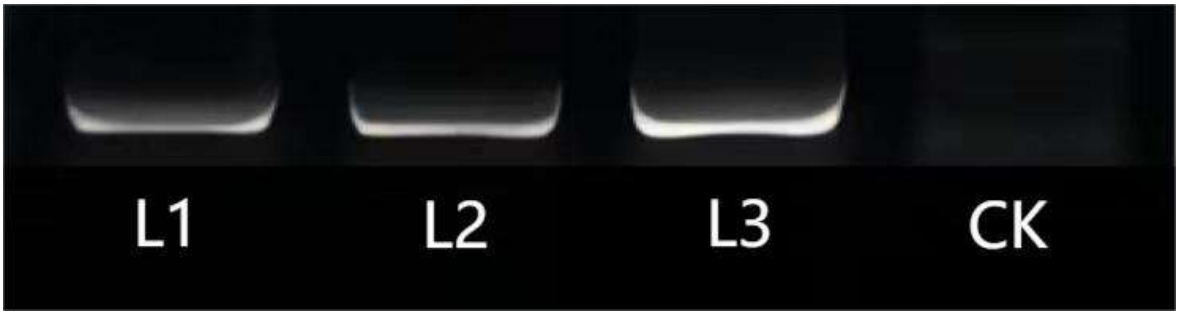


Figure 3.3 – *Bar* gene PCR assay

3.3.2 Real-Time PCR assay

Leaf samples from transgenic and non-transgenic maize were assayed using fluorescence quantitative PCR and semi-quantitative PCR to determine the expression level of the *ZmSUS1* gene. The results showed that the *ZmSUS1* gene showed efficient expression in transgenic lines L1, L2 and L3 and the expression level was much higher than the control. The relative expression of *ZmSUS1* gene was substantially elevated by about 443%-1175% compared to the control.(Fig. 3.4)

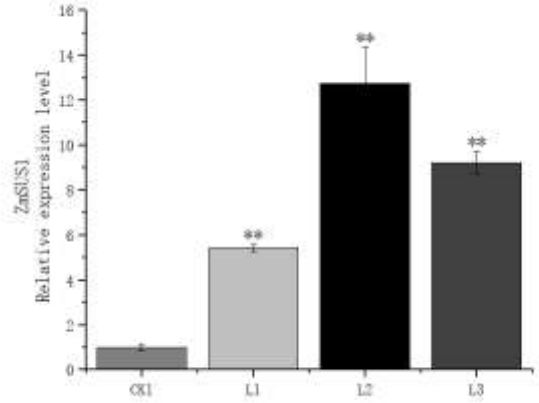


Figure 3.4 – Relative expression of the *ZmSUS1* gene

3.4 Overexpression of ZmSUS1 improves drought tolerance in seedling maize

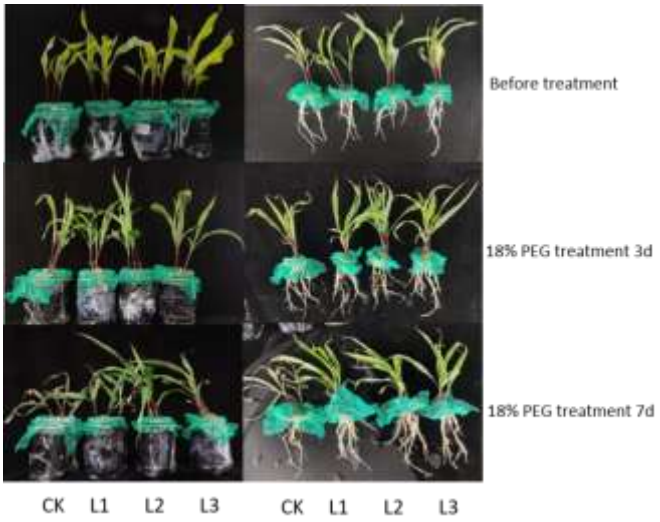
The water content of plant leaves is a key indicator of the degree of water loss under drought stress, which intuitively reflects the drought response ability of plants. Meanwhile, proline, soluble sugar and ABA are also key parameters for plant response to environmental stress. In order to deeply investigate the effect of overexpression of *ZmSUS1* gene on the drought resistance of transgenic maize, three stable transgenic T3 generation maize lines (L1, L2, L3) and non-transgenic negative control line CK were selected in this study, and the phenotypic changes were observed under drought stress conditions, and relevant physiological and biochemical indexes (proline, soluble sugar and ABA) were detected at the same time.

3.4.1 Phenotypes of drought-treated plants

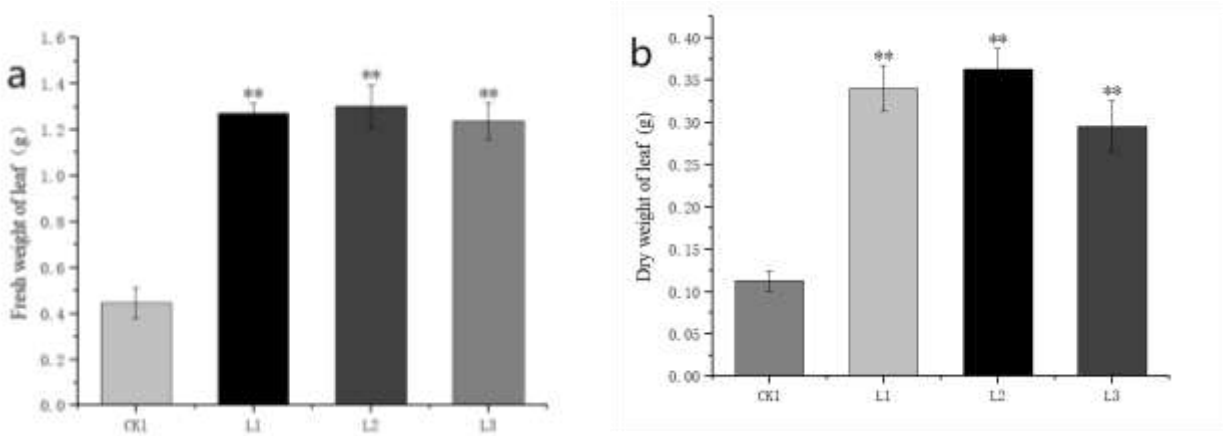
After drought treatment, the transgenic maize plants had a healthier overall phenotype compared to the control, although they also showed wilting. After seven days of drought treatment, the transgenic maize successfully recovered and continued to grow after re-watering, while the control maize recovered poorly and some plants even died.(Fig. 3.5)

Figure 3.5 – Drought treatment phenotypes

3.4.2 Relative water content and fresh and dry weight after drought treatment



After drought treatment, control plants and transgenic plants showed significant differences in several physiological indicators. As shown in Figure 3-5a, compared to the control, the transgenic group of maize increased the fresh weight of the aboveground part by 169% to 192%. The aboveground dry weight of the transgenic maize was increased by 161.5%-221.5% compared with the CK plants. Also the relative water content of transgenic plants was higher than that of the control group by about 16.1%-19.7%. These differences indicated that maize transgenic with



ZmSUS1 gene showed stronger physiological adaptability and stability under drought stress.(Fig. 3.6-Fig3.7)

Figure 3.6 – Leaf dry and fresh weight

Note: Figures 3-6a and 3-6b show the fresh and dry weight contents of leaves of transgenic group and control group after drought treatment, respectively.

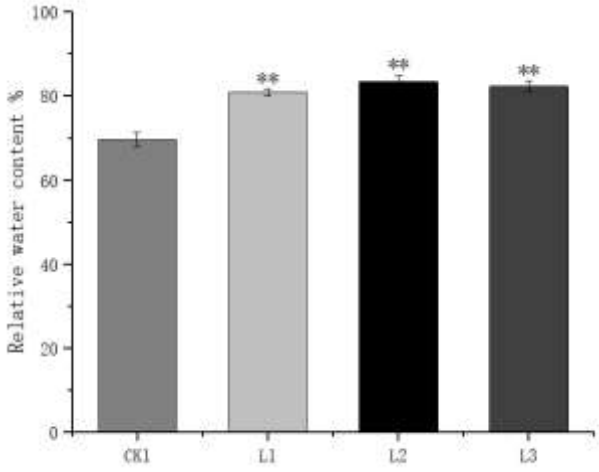


Figure 3.7 – Relative water content of leaves

3.4.3 ABA and proline content

In this study, abscisic acid (ABA) and proline contents of maize in the transgenic and control groups were measured after drought treatment. It was found that the ABA and proline contents of maize in the transgenic group increased significantly in response to drought stress compared with those in the control group, with the ABA content increasing significantly by 22% to 33%, and the proline content also increased by about 13% to 34%.

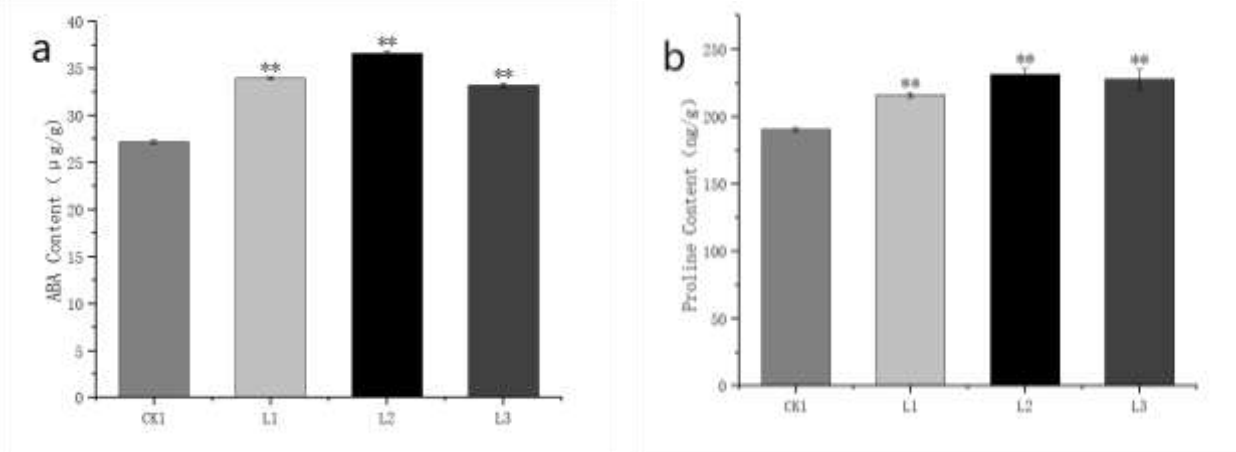


Figure 3.8 – ABA and proline content

Note: Figures 3-8a and 3-8b show the fresh and dry weight contents of leaves of transgenic group and control group after drought treatment, respectively.

3.4.4 Soluble sugar content

Changes in soluble sugar content have become a key indicator of plant response to drought stress and play an important role in osmotic adjustment. As shown in Figure 3-8b, there was a significant increase of about 25.9%-60% in the content of soluble sugars, the main substance for drought resistance in maize.

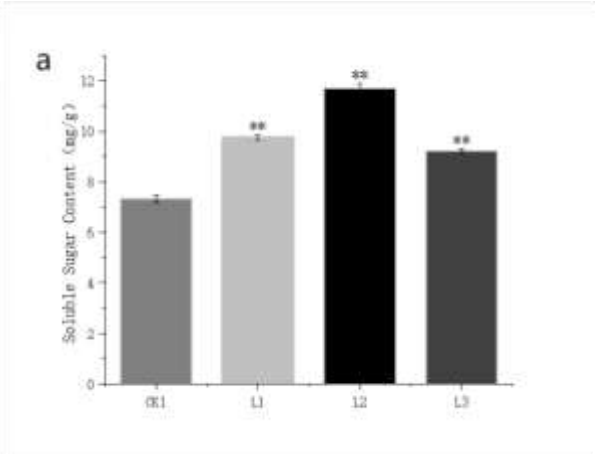


Figure 3-9 Soluble Sugar Content

After 7 consecutive days of natural drought treatment, the control plants showed significant leaf wilting and chlorosis, with leaf loss of water, dull colour and fragile texture, demonstrating severe drought stress effects. In contrast, maize plants overexpressing the *ZmSUS1* gene showed less wilting of the leaves, which still retained a certain degree of moisture and vigour, showing strong drought resistance.

After rewatering, maize plants overexpressing the *ZmSUS1* gene showed excellent recovery ability, and the plants resumed growth two days after rewatering. In contrast, the control plants had a lower degree of recovery, and some plants even died and could not return to normal growth. Compared with the control plants, the drought-treated transgenic plants showed significant physiological advantages. The transgenic plants were significantly higher than the control plants in key indicators such as fresh weight, dry weight and relative water content of the aboveground parts. ABA and free proline accumulation in the leaves of transgenic plants also showed a significant increase relative to the control.

4.1 Discussion

In this paper, we constructed the SUS gene family evolutionary tree and analysed the affinities of SUS genes in different plant species. At the same time, through the analysis of the *ZmSUS1* promoter sequence, various regulatory elements in the promoter region of the gene were identified, and the roles and functions of these regulatory elements were initially understood, and it was found that there were many promoter cis-acting elements related to drought stress in this gene.

Using Agrobacterium transformation method, we successfully constructed transgenic maize lines overexpressing the *ZmSUS1* gene, and found that *ZmSUS1* was induced to be expressed by drought stress, and the drought-treated transgenic plants possessed a faster recovery ability than the control plants after rewatering, and showed better growth phenotypes, higher relative water content, dry weight and fresh weight under drought conditions.

Drought stress can promote the improvement of drought-related indexes such as proline and ABA in maize transgenic with *ZmSUS1*. ABA can regulate the water balance of plant leaves, reduce water evaporation by regulating the stomatal switch, and promote water uptake by the roots, which can help the plant to cope with drought stress. Proline acts as an osmotic regulator and helps to maintain cellular osmotic balance, thereby enhancing plant stress tolerance. Proline is also involved in scavenging free radicals, reducing cell acidity, and acting as a metal chelator. Proline also acts as an anti-dehydrating agent, reduces ammonia toxicity from protein hydrolysis, stores nitrogen and carbon, and provides a respiratory substrate and carbon source for recovery after adversity is lifted.

Drought stress significantly increased the soluble sugar content of maize transfected with the *ZmSUS1* gene, increased the intracellular osmotic pressure, maintained the water balance of the cells, reduced the damage to cell membranes and proteins caused by drought stress, and elevated the photosynthetic rate of the plant, which further enhanced the drought tolerance of the plant.

In summary, the *ZmSUS1* gene significantly enhanced the drought tolerance of overexpressing *ZmSUS1* plants by promoting sucrose catabolism and increasing the

content of soluble sugars, proline, and abscisic acid (ABA), and maintained high leaf water content under drought conditions. This study enriches the understanding of the role and physiological and molecular mechanisms of *ZmSUS1* gene in abiotic adversity response, and enriches the innovative pathway for genetic engineering to breed new drought-tolerant crops.

4.2 Outlook

Although this experimental study has proved that *ZmSUS1* gene plays an important role in maize response to drought, further research on the specific mechanism of this gene is still needed.

This study has demonstrated that overexpression of *ZmSUS1* gene increased the content of ABA, proline and sucrose in transgenic plants. In future research work, the specific mechanism of *ZmSUS1* overexpression to increase the content of ABA, proline, and sucrose can be further investigated through different signalling pathways and physiological and biochemical processes, such as the ABA signalling pathway, proline metabolism pathway, and sugar metabolism pathway.

In future studies, transcriptomics studies and data analysis can be used to reveal the functions and roles of *ZmSUS1* gene expression in different tissues and at different stages of growth and development.

This study preliminarily demonstrated that the promoter region of the *ZmSUS1* gene contains many action elements related to drought response, such as DREB, STRE , MBS, CGTCA-motif, and ABA response elements. The in-depth study of these loci will help to reveal the expression pattern of *ZmSUS1* gene in maize and the mechanism of interaction with other genes, which can provide a more precise understanding of the role of *ZmSUS1* gene in drought resistance of plants and provide more accurate targets for genetic engineering breeding.

In addition, this study only analysed drought resistance in maize at the seedling stage, and in the future, drought resistance analysis can be carried out in maize at more developmental stages to comprehensively assess the role of the *ZmSUS1* gene. Meanwhile, studies on the interaction of this gene with other drought resistance

related genes can also be conducted to further reveal the position and role of *ZmSUS1* in the drought resistance signalling pathway.

CONCLUSIONS

1. Constructed an evolutionary tree of the SUS gene family, and analysed the affinities of SUS genes in different plant species.
2. Analysed the promoter sequence of ZmSUS1, identified various regulatory elements in the promoter region of the gene, and gained a preliminary understanding of the roles and functions of the regulatory elements
3. The ZmSUS1 gene promotes sucrose catabolism and increases the content of soluble sugars, proline and abscisic acid (ABA), which significantly enhances the drought tolerance of overexpressed ZmSUS1 plants and maintains high leaf water content under drought conditions.
4. In this study, using genetic engineering, we constructed maize lines overexpressing the ZmSUS1 gene and proved that the trans-ZmSUS1 gene improved the drought tolerance of maize, which provides an important reference for the use of genetic engineering technology to improve the drought tolerance of crops.

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