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 **Analysis of Cold Resistance of Different Wheat Varieties and Cloning of Cold Resistance Genes**
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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APPROVE
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ASSIGNMENTS
FOR THE QUALIFICATION THESIS
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scientific supervisor Tetiana Shcherbatiuk, Dr. Sc., Prof.

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SUMMARY

Analysis of Cold Resistance of Different Wheat Varieties and Cloning of Cold Resistance Genes. – Manuscript.

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Wheat is widely planted in China, and its production is very important for the sustainable supply of grain in China. In the north of China, the lowest temperature in winter can reach -44.3℃, and cold in spring often occurs. With the continuous increase of extreme climate, the ecological conditions are getting worse, and cold has become one of the main factors restricting the development of wheat. In order to reduce the harm of abiotic stress to wheat industry, besides improving environmental conditions, a more effective method is to explore key genes through genetic engineering breeding and improve the adaptability of plants to adversity. The cold adaptation of wheat is realized by activating a group of cold response genes to encode cryoprotective proteins that protect cells from cold-induced damage. Currently, there is increasing evidence that CBF and the ICE1 transcriptional cascade pathway play important roles in regulating freezing resistance in plants. In this study, several wheat varieties with different cold resistance were selected for cold and chilling resistance experiments, and two varieties with different cold resistance were screened out (Tai 113 and C8178). Then follow-up experiments were conducted, mainly using RT-PCR to analyze the expression of some pairs of common cold-resistant genes at the transcriptional level, and the screened cold-resistant genes (CS4A02G433200 and CSIA02G137500) were cloned to provide genetic resources for cold-resistant breeding in wheat.

Key words: wheat, Cold stress, Gene cloning, CBF, ICE1.

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INTRODUCTION

This paper summarizes recent advances in wheat cold tolerance research and the application of these findings in wheat production and breeding practices. With the intensification of global climate change and the frequent occurrence of extreme weather events, the impact of cold stress on wheat growth and production has become more and more significant. Therefore, improving the cold tolerance of wheat has become an important challenge for current wheat breeding and production.

Firstly, we explored the cold tolerance of different varieties of wheat in depth through a variety of methods, such as germination cold tolerance experiments, soil cultivation method of planting and seedling cold tolerance experiments. These experiments not only involved the determination of phenotypic data, but also combined with gene expression analysis, thus revealing the genetic basis and molecular mechanism of cold resistance in wheat. The results of these studies indicate that there are significant differences in cold resistance among different varieties of wheat, and that such differences are closely related to the expression levels and regulatory mechanisms of genes.

In order to further reveal the molecular mechanism of cold resistance in wheat, we successfully identified a number of genes related to cold resistance using RNA extraction, cDNA library construction and qRT-PCR validation. The expression changes of these genes in different varieties of wheat showed a high degree of consistency with their cold resistance performance, which provides an important theoretical basis and genetic resources for breeding cold resistance in wheat.

During the in-depth study of these cold-resistant genes, researchers also discovered their key roles in the response to cold stress in wheat. These genes enhance the survivability and adaptability of wheat in low-temperature environments by regulating its physiological and metabolic processes. In addition, the researchers have successfully cloned some of the cold-resistance genes by using modern biotechnological means, which lays the foundation for subsequent gene function studies and applications.

In addition to research at the gene level, this paper also summarizes the research progress in wheat photosynthetic characteristics, cold resistance mechanism and genetic basis. These studies not only contribute to a deeper understanding of the physiological and ecological basis of cold resistance in wheat, but also provide theoretical support and practical guidance for the improvement of wheat cultivation technology and the selection and breeding of excellent varieties. For example, by optimizing wheat cultivation measures such as planting density and fertilizer application, the cold resistance and yield of wheat can be effectively improved.

In addition, this paper introduces the key genes in the plant cold response mechanism, as well as the characterization and expression patterns of ICE-CBF-COR genes in wheat. These genes play important roles in plant cold stress response by regulating the expression of downstream genes, which in turn affects the cold resistance of plants. The in-depth study of these key genes not only helps to reveal the molecular mechanism of plant cold response, but also provides new perspectives and strategies for cold tolerance breeding. It is worth mentioning that this paper also focuses on other issues in wheat production practice, such as the research and development of high-yielding wheat varieties and supporting cultivation techniques, the application of hydroponically grown wheat seedlings in animal husbandry production, and the effect of potash fertilizer on the effect of hydroponics on wheat seeds. These studies not only demonstrate the wide application of modern agricultural science and technology in wheat production, but also provide new ideas and methods for the sustainable development of wheat production.

In summary, this paper summarizes the recent progress of wheat cold resistance research and its application in production and breeding practice. Through in-depth research on the physiological and ecological basis and molecular mechanism of wheat cold resistance, combined with the application of modern biotechnological tools, researchers have provided important theoretical basis and practical guidance for wheat cold resistance breeding and production. These research results not only help to cope with the challenges of climate change on wheat production, but also inject new impetus for the sustainable development of the wheat industry.

However, despite the remarkable progress we have made in wheat cold resistance research, there are still many issues that need to be addressed. For example, how to further improve the expression efficiency and stability of cold resistance genes? How can these genes be effectively applied in wheat breeding practices? In addition, we need to strengthen research on the association between cold resistance and other traits in wheat in order to breed new wheat varieties that are both cold resistant and high yielding and high quality.

In the future, we can continue to promote the research and application of cold resistance in wheat from the following aspects: first, to strengthen the mining and functional verification of cold resistance genes, to provide more excellent genetic resources for breeding cold resistance in wheat; second, to deepen the understanding of the molecular mechanism of cold resistance in wheat, and to reveal more genes and signaling pathways related to cold resistance; third, to explore the balance and synergy between cold resistance and other traits, and to breed new wheat varieties with excellent integrated traits; third, to explore the balance and synergy between cold resistance and other traits, and to breed new wheat varieties with excellent integrated traits. Third, to explore the balance and synergy between cold tolerance and other traits, so as to cultivate new wheat varieties with excellent overall traits; fourth, to strengthen international cooperation and exchange of research on cold tolerance of wheat, so as to jointly respond to the challenges of global climate change on wheat production. Wheat cold resistance research is a field full of challenges and opportunities. Through in-depth research and practical exploration, we are expected to provide more effective theoretical support and technical means for cold-resistant wheat breeding and production, and contribute to the sustainable development of the wheat industry.

The relevance of the topic is analysis and cloning of unknown pit-cooling genes in wheat by using known molecular biology methods.

The purpose of the study is to study wheat cold resistance genes at the transcriptional level.

The objectives of the study provide theoretical support and genetic resources

for wheat cold resistance breeding.

The objects of the study are cold stress response of wheat, identification of cold resistance related indexes in wheat and cold resistance related genes in wheat

The subject of the study are wheat varieties studied were Tai 113 and C8178. And the genes studied mainly consisted of genes related to cold resistance in wheat, including CBF and ICE1 (two key cold resistance gene families).

Research methods Bioinformatics, qRT-PCR.

The scientific novelty discovery and cloning of unreported genes.

The practical significance of the results obtained is Used to create wheat that is more cold-tolerant.

CHAPTER 1
LITERATURE REVIEW

1.1 Wheat cultivation in China

Wheat is a major cereal for meeting global food demand [1]. In major wheat-growing regions of the world, such as China, the United States, Europe, and Australia, low temperatures often hamper its growth and yield [2]. Cold conditions also limit active root water uptake, leading to stem water deficit and triggering drought stress [3], which inevitably impairs wheat growth. In recent years, wheat growing areas in the Yellow River and Huaihe River in China have frequently suffered from inverted spring cold, which disrupts normal growth and reduces the final yield of wheat. The frequent occurrence of cold damage in this region resulted in a significant reduction of final grain yield by 30-50% in severe cases, affecting nearly 42% of the wheat sown area [4].

Low temperature is a frequently encountered environmental stress during the plant life cycle and has significant effects on crop growth, development, yield and quality. Cold stress, including cold above 0 °C and freezing below 0 °C [5], poses severe challenges to plant growth and development. To cope with these challenges, plants have evolved a variety of signal sensing and response mechanisms, resulting in complex changes in morphology, biochemistry and physiology. Currently, research on cold tolerance in wheat covers multiple levels, including morphology, cytology, physiology and biochemistry, and molecular biology. The study of winter wheat cold resistance is not only related to the division of winter and spring wheat areas, but also an important basis for seed selection, introduction and cultivation technology determination [6], and most importantly, it is directly related to national food security and other issues [7].

1.2 Cold stress response in wheat

Cold damage is the damage to plant production and development caused by low temperatures above freezing point [8]. It usually occurs during the gestation period of wheat growth and mainly affects seedling growth and grain yield. When crops are affected by cold, rice and maize often appear in the form of rotting seeds, hard or even dead seedlings, as well as wheat, where the ears are damaged in whole or in part, as evidenced by tasseling or delayed empty ears. Global warming has led to warm winters in recent years, and as wheat enters the tassel stage, its high water content and soft tissues make it more sensitive and less resistant to sudden low temperatures. Frost damage is damage caused by freezing in plant cells or tissues as a result of low temperatures below freezing point, and can be divided into winter frost damage and spring frost damage [9]. The former, with loss of cellular stress, chlorophyll destruction, and change of leaves from green to brown, occurs with low probability and weak extent, with underground parts almost unaffected and low seedling mortality. The latter is affected by the lowering of chilling temperatures and the degree of damage varies according to the stage of development.

At the stage of nutritive growth, the seedling stage of wheat where the growth of nutritive organs such as roots and leaves are mainly carried out. If this stage is subjected to cold stress, it results in poor malting and slow seedling growth, thus affecting the yield and quality of wheat in later stages. In the stage of concurrent nutritive and reproductive growth, wheat carries out the differentiation and development of spikes on the one hand, and continues to grow roots, leaves and tillers on the other. If this stage is subjected to cold stress, it will affect the photosynthesis of wheat, leading to a decrease in dry matter accumulation, which in turn affects the yield and quality of wheat. Moreover, low temperatures can also cause yellowing and dieback of leaves where wheat growth is severely affected. In the reproductive growth stage, the growth center of wheat is transferred to the reproductive organs, and at this time, if cold stress is encountered, it will have a serious impact on the flowering and fertilization of wheat and the formation of seeds, resulting in a decrease in the grain weight of wheat and a reduction in yield [10]. In

addition, vernalization requirements may not be fully met, resulting in delayed flowering and exposing the plant to different biotic and abiotic stresses [11].

Overall, the main effects of cold stress on wheat are that it can lead to stunted growth and development, reduced enzyme activity for yield and quality, damage to the membrane system, and cellular water loss [12].

1.3 Cold resistance related genes in wheat

When wheat is subjected to low-temperature stress, it undergoes a delicate physiological response process. First, the wheat plant acutely senses the decrease in external temperature, which is the starting point of its self-protection mechanism. Next, this cold signal is rapidly transmitted within the plant, ensuring that the information is quickly and accurately transmitted to all corners of the cell. Ultimately, this series of signals induces the expression of downstream genes that encode proteins that help wheat adapt to and withstand cold temperatures. Transcription factors play a crucial role in this complex and precise response pathway [13]. They are able to bind to the promoters of specific genes and regulate the transcriptional activity of these genes, thus affecting the response of wheat to low temperature.

Among them, the key role of CBF and ICE genes in black wheat crops involved in abiotic stress response has been demonstrated [14]. ICE (inducer of CBF expression), a member of the MYC family of transcription factors, is characterized by its ability to induce CBF expression. The main structural feature of ICE is that it shares the highly conserved CBF (C-repeat-binding factor)/DREB transcription factors belong to the AP2/ERF multigene family, which is a key regulator region for freezing tolerance in plants [15]. ICE factors are positive regulators of CBF expression upstream of the low-temperature signaling pathway. CBF transcription factors are independent proteins involved in stress signaling [16], and are essential for cold domestication and freezing tolerance in plants [17]. CBF is a key component of the ICE-CBF-COR pathway, which is rapidly activated after brief exposure to low innocuous temperatures, has been isolated and characterized in Gramineae in cold-sensitive (rice and maize) or frost-tolerant species (wheat, barley, and rye) for a

variety of elements of the family [18], and it is one of the most relevant mechanisms for activating the adaptive responses to cold and drought in wheat, barley, and rye.

The ICE-CBF-COR pathway is activated through an increase in intracellular Ca^{2+} is activated to concentrate through rigidified or ligand-activated channels in the plasma membrane. After calcium influx into the cytoplasm and binding via calcium sensors such as calmodulin, a calcium-binding protein (CBP)-based signaling cascade is initiated to target ICE (inducer of CBF gene expression) transcription factors that upregulate the CBF genes [19]. The ICE transcription factors belong to the MYC family and the MYC subfamily of the bHLH (basic helix-loop-helix) and have been referred to as CBF positive expression regulators [20] and are thought to act upstream of the low-temperature signaling pathway [21]. The ICE-CBF-COR pathway cascade response results in the activation of effector genes that alter plant metabolism and confer cold tolerance [22]. Understanding the complex mechanisms and regulation of the CBF gene clusters carried by the homologous chromosomes of cold-resistant genes in plants in wheat has great potential for genetic improvement of wheat.

1.4 Identification of Cold Resistance Related Indicators in Wheat

Cold tolerance in wheat, as a key indicator of its adaptability and yield, is particularly important in cold northern regions. In order to comprehensively and accurately assess this trait, researchers need to adopt a series of refined identification methods. The most basic of these methods is visual assessment, which is used to initially determine the cold tolerance of wheat by observing its growth status, leaf color change and other external characteristics under cold conditions. In addition, field counting is also an important identification tool to quantitatively assess the cold tolerance of wheat varieties by counting the number of wheat plants affected by frost damage. These characterizations not only provide an important basis for wheat breeding, but also provide guidance for wheat growers to select appropriate varieties in cold regions.

(1) Visual assessment

In assessing the cold resistance of wheat seedlings, we first carried out a detailed classification and analysis based on their growth characteristics and morphological features. Specifically, we categorized wheat seedlings into three well-defined classes based on their growth postures in cold environments in order to more accurately evaluate the strength of their cold resistance.

First, we defined the third level of cold resistance type as prostrate. Wheat seedlings in this class grow almost completely close to the ground when faced with a cold climate, showing an advective growth attitude. This growth attitude not only reduces the light area of the leaf surface and affects photosynthesis, but more importantly, it significantly increases the portion of the seedling that is in contact with the ground, exposing more leaf tissues to lower temperatures and thus exacerbating the damage caused by low temperatures to the seedling. As a result, creeping wheat seedlings typically exhibit weaker cold resistance.

Second, we defined a secondary cold resistance type as semi-prostrate. Wheat seedlings in this category remain off the ground when exposed to low temperatures, although the tip portion of the leaf touches the ground, the main portion of the leaf blade remains off the ground. This semi-prostrate growth not only ensures a certain light area and maintains the efficiency of photosynthesis, but also reduces the contact area of the leaf blade with the ground to a certain extent, thus alleviating the damage caused by low temperature to the seedlings. As a result, wheat seedlings of semi-prostrate type showed a moderate level of cold resistance.

Finally, we defined the first level of cold resistance type as erect type. Wheat seedlings in this category were able to maintain their leaves in a fully upright growth position under cold conditions. This upright type of growth not only ensures the maximum light area of the leaf blade, which is beneficial for photosynthesis, but also minimizes the contact of the leaf blade with the ground, which reduces the damage of low temperature to the seedlings. As a result, wheat seedlings of the upright type usually show a strong resistance to cold.

(2) Field count identification [23].

This method is based on the changes in the number of stems in the field before and after overwintering, and determines the number of dead stems after freezing treatment, so as to assess the cold resistance. This method is not only characterized by intuition and operability, but also the results can more accurately reflect the cold resistance performance of crops in actual production. We evaluated the cold resistance of Tai 113 and C8178 by the field counting method.

Although the field counting method is simple and easy to use, some details need to be paid attention to in the process of implementation. For example, we should try to avoid missing or repeated counts to ensure the accuracy of the data. At the same time, the effects of humidity and the size of the growing environment on the cold resistance of the crop should also be taken into account in order to assess the cold resistance of wheat in a more comprehensive manner.

Conclusions to chapter 1

1. These two assays play an indispensable role in the assessment of cold tolerance in wheat. These determinations not only help us to get a comprehensive understanding of the physiological conditions of wheat in cold environments, such as growth rate, leaf changes, etc., but also reveal the potential characteristics of its nutritional quality and resilience. Through these careful observations and statistics, we can more accurately assess the adaptability and yield potential of wheat varieties and provide a solid scientific basis for wheat cultivation and breeding. Of course, due to the diversity of experimental purposes and conditions, the specific measurement methods and indicators may need to be adjusted and optimized according to the actual situation to ensure the accuracy and reliability of the assessment results.
2. In recent years, the frequent occurrence of extreme climatic events has significantly affected agricultural production, with low-temperature freezing being one of the main factors leading to a significant decline in wheat yield. In view of the complexity of the cold resistance mechanism of wheat, we

need to deeply analyze its response characteristics under cold stress from diversified perspectives and levels.

3. In this study, several wheat varieties with different cold resistance were selected for cold and chilling resistance experiments, and two varieties with different cold resistance (Tai 113 and C8178) were screened out. Then subsequent experiments were conducted, mainly using RT-PCR to analyze the expression of some pairs of common cold resistance genes at the transcriptional level [13], by which key differentially expressed genes were identified and key candidate genes were targeted. Eventually, we expect to elaborate the genes that play important roles in cold and cold resistance in wheat, which not only has important theoretical value for enhancing the cold resistance of wheat, but also provides a strong scientific basis for agricultural production practice.

CHAPTER 2
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Materials for testing

In the current research, we focused on comparing the cold tolerance of two wheat varieties, Tai 113 and C8178, through hydroponic growth experiments. Tai 113, a novel winter wheat variety, was jointly bred by the Institute of Crop Science, Shanxi Academy of Agricultural Sciences, and the Biotechnology Research Center of Shanxi Academy of Agricultural Sciences [24]. Its development began in 2003, through sexual hybridization of Jinmai 66 and Baofeng 104. This variety was selected due to its promising agronomic traits and potential for cold tolerance. On the other hand, C8178 is a novel wheat variety developed through hybridization in our laboratory. The choice of this variety was motivated by its potential to exhibit superior cold tolerance, which could be valuable for breeding programs aimed at producing wheat varieties that can withstand harsher environmental conditions.

To conduct this study, we carefully selected seeds of both Tai 113 and C8178, ensuring they were large, full, and of uniform quality. These seeds were then sown on December 26, 2023, in the experimental field, with twelve replications set up for each variety. The first sampling was performed on March 15, 2024, where the seedlings were exposed to a cold treatment of 4 °C for 24 hours. This treatment was chosen to simulate mild cold stress conditions that wheat plants may encounter during the early stages of winter. For the second sampling, conducted on the same day, the seedlings were exposed to a more severe cold treatment of -18 °C for 3 h. This extreme temperature was chosen to assess the cold tolerance of the two varieties under more challenging conditions. Finally, for the third sampling, the seedlings were thawed at 4 °C for 24 h on March 16, 2024. This step was crucial to evaluate the recovery ability of the seedlings after cold stress exposure.

From each sampling, two replicate groups of seedlings were selected for further analysis. These seedlings were quickly frozen in liquid nitrogen to preserve

their physiological state and labeled clearly for identification. They were then stored at -80 °C for subsequent experiments, which would involve gene expression analysis, biochemical assays, and other techniques to gain a deeper understanding of the cold tolerance mechanisms in Tai 113 and C8178.

2.2 Test methods

2.2.1 Hydroponic cultivation of wheat seeds

In this study, we employed a rigorous protocol to assess the cold tolerance of two wheat varieties, Tai 113 and C8178. Starting with the selection of healthy, pest and disease-free seeds, we ensured that 150 seeds each of both varieties were chosen for the experiment. The use of purified water as the germination medium was crucial to eliminate any potential contaminants that could affect the germination process [25]. Before germination, the seeds were thoroughly washed with water and gently stirred to ensure that any impurities on their surfaces were removed. To further ensure sterility, the seeds were sterilized with alcohol, minimizing the risk of fungal or bacterial growth during the germination process. To promote water absorption and germination, the seeds were soaked in water for a few minutes. This step not only cleaned the seeds but also helped them to absorb water, which is essential for germination. The germination environment was carefully prepared. The soaked seeds were then placed in a fixing basket that contained an appropriate amount of water. Each basket held approximately 50 seeds, ensuring that there was adequate spacing between the seeds to prevent overcrowding [26].

The baskets were then covered and placed in a 25 °C constant temperature incubator. To mimic natural conditions, the incubator was set to a 16-h light and 8-h dark cycle. Additionally, to maintain a moist environment, a small amount of sterile water was sprayed into the incubator at the same time every day.

Once the malt had grown to a length of 1-2 cm, it underwent a series of cold treatments. First, the malt was pretreated in a 4 °C refrigerator for 10 d. This gradual exposure to low temperatures allowed the malt to acclimate to the colder environment.

Following this, the malt was frozen in a -20 °C freezer for 12 h. This harsher cold treatment tested the malt's ability to survive extreme cold conditions.

After freezing, the malt was moved back to a 4 °C refrigerator to thaw for 24 h. This step allowed the malt to recover from the cold stress and resume growth. Finally, the malt was kept at 4 °C for another 24 h to ensure that any lingering effects of the cold treatments had subsided.

During each stage of the cold treatments, the total number of malt and the number of surviving melt were carefully recorded. Specifically, these measurements were taken on the 10th day of pretreatment at 4 °C, the 12 h of treatment at -20 °C, and the 12 h of thawing treatment at 4 °C. These data provided crucial insights into the cold tolerance of the two wheat varieties.

Based on the recorded data, we calculated the survival rate of the malt after each treatment. This metric allowed us to directly compare the cold tolerance of Tai 113 and C8178 and assess which variety performed better under cold stress conditions.

2.2.2 Growing wheat by soil culture method

(1) Preparation of soil: As the basis for crop growth, the quality of soil directly determines the growth of crops. Therefore, before planting, we need to carefully prepare the soil. First of all, the selection of nutrient soil, this soil can provide adequate nutritional support for crops. Second, the soil is mixed, this step not only helps to improve the physical structure of the soil, make it looser, increase the permeability of the soil, which is conducive to the respiration and growth of the crop root system; it also improves the water retention capacity of the soil, to ensure that the crop in the process of growth to obtain sufficient water. The soil after mixing should maintain the appropriate humidity and temperature to create good environmental conditions for the growth of crops [27].

(2) Selection and treatment of seeds: The quality of seeds is crucial to the growth of crops. In order to improve the germination rate and growth rate of seeds, we need to strictly select and treat seeds before sowing. First of all, we need to select

healthy, full, disease and pest-free seeds, which is the basis for ensuring seed quality. High-quality seeds not only have a higher germination rate, but also reduce the occurrence of pests and diseases, and improve the yield and quality of the crop.

After selecting seeds, we need to carry out seed soaking treatment. Soaking seeds in a growth promoter for a period of time is a step that can accelerate seed germination, improve germination rates, and promote robust seedling growth. However, during the seed soaking process, we need to be careful to control the soaking time and the concentration of growth promoter. Too long soaking time or too high concentration of growth promoter may cause unnecessary damage to the seeds and affect their germination and growth.

In addition, it is also crucial to sterilize the seeds. Through sterilization, we can effectively kill germs and insect eggs on the surface of seeds and reduce the occurrence of pests and diseases. Common sterilization methods include ultraviolet light irradiation, hot water immersion and so on. When choosing disinfection methods, we need to choose according to the characteristics of the seeds and the actual situation to ensure the disinfection effect while avoiding damage to the seeds.

(3) Sowing: Sowing is an important part of the planting process, which determines the planting density and growing space of the crop. Before sowing, we need to determine the appropriate sowing depth and density according to the growth characteristics of the crop and soil conditions. Generally, treated seeds should be sown at a depth of 3-5 cm below the soil surface [28]. This depth can ensure that the seeds are in full contact with the soil, which is favorable for the seeds to absorb water and nutrients in the soil. At the same time, it avoids sowing too deep resulting in seed germination difficulties.

During the sowing process, we need to pay attention to maintaining the uniformity and consistency of sowing. This can be achieved by using a seeder or sowing by hand. Make sure that each seed has enough room to grow so that it can absorb nutrients and sunlight in a balanced way as it grows. After sowing, we also need moderate soil compaction and watering to promote seed germination and growth.

(4) Field management: Reasonable water and fertilizer management is crucial to the growth of wheat during the growing period. We need to water the wheat every other day to ensure the water and nutrients needed for wheat growth. Meanwhile, in order to avoid damage to wheat caused by spring winds, we need to move the pots to a well-lit place indoors to ensure that the wheat can carry out normal photosynthesis.

In addition, weeding and pest control are not to be neglected in field management. Weeds and pests will seize the nutrients and growing space of wheat, affecting its growth and yield. Therefore, we need to regularly weed and carry out pest control to minimize the impact of these unfavorable factors on wheat growth. In the process of weeding and pest control, we need to pay attention to the use of environmentally friendly and safe methods to avoid harming the environment and human health. [29].

(5) Seedling Cold and Cold Resistance Experiments:In order to evaluate the cold tolerance of two wheat varieties, Tai 113 and C8178, a series of controlled experiments were conducted. Specifically, three pots of Tai 113 and three pots of C8178, all exhibiting similar growth height and tiller number, were selected for the experiments. Each variety was divided into three experimental groups, with one pot serving as the control group and the other two pots representing experimental groups.

The first group, the control group, remained in stable room temperature conditions throughout the experiment. This group served as a baseline for comparing the cold tolerance of the other groups. The second group, the first experimental group, underwent a specific cold stress protocol. Initially, the seedlings were pretreated in a 4 ℃ incubator for 24 h to induce a gradual decrease in temperature. Following this pretreatment, the seedlings were then transferred to a -18 ℃ incubator for a duration of 3 h, exposing them to a more extreme freezing environment. This rapid drop in temperature was designed to test the seedlings' ability to survive and recover from a severe cold shock. The third group, the second experimental group, underwent a different cold stress protocol. Like the second group, the seedlings were pretreated in a 4 ℃ incubator for 24 h. However, after the pretreatment, they were not subjected to the freezing temperature but instead were kept in the 4 ℃ incubator continuously.

This group served to evaluate the seedlings' response to a sustained low temperature stress.

All experimental groups were maintained under identical photoperiod conditions, with 16 h of light and 8 h of darkness each day. To ensure adequate hydration, the same amount of tap water was poured into each pot every evening, every two days. The seedlings were carefully monitored, and phenotypic changes, such as discoloration, wilting, or any other visible signs of stress, were observed and recorded daily. These observations provided crucial insights into the cold tolerance of the two wheat varieties under different stress conditions.

2.2.3 Determination of phenotypic data

When evaluating cold tolerance in wheat, plant height and stem thickness serve as crucial physical indicators that provide valuable insights into the plant's growth potential, robustness, and adaptability. These measurements are not just arbitrary numbers; they are indicators that reflect the inner vitality and resilience of the plant.

(1) Plant height: Plant height is a key indicator of a wheat variety's growth potential and adaptability. As the plant grows, the height increases, reflecting its ability to absorb nutrients and sunlight efficiently. By regularly measuring and recording the growth height of wheat samples, we can track the plant's growth rate and growth trend at different stages. This data allows us to assess the variety's potential for high yields and its adaptability to varying environmental conditions. For instance, a wheat variety with a consistently high growth rate may indicate a strong potential for high yields, while a variety that maintains stable growth under stress conditions may demonstrate good adaptability.

(2) Stem thickness: It is a critical indicator of the robustness and stability of wheat plants. A thick and sturdy stem provides better support for the plant, enabling it to withstand external forces such as wind and rain. Moreover, a thick stem indicates a healthy and vigorous plant, which is more likely to produce high yields. By measuring the stem thickness of wheat samples, we can understand the plant's supportive ability and resistance. This is crucial for assessing the yield and quality of

wheat, as a weak stem can easily be damaged, affecting the plant's growth and development.

(3) Photographing method: To accurately record the growth of wheat and compare the performance of different varieties, we employ a photographic method. This approach allows us to visually capture the growth status of wheat samples and compare them over time. To ensure clear and true colors in the photos, we select wheat samples with similar numbers of tillers and use a black background with a soft and concentrated light source from outside. This setup eliminates any potential distortions or reflections, ensuring that the photos accurately represent the true colors and growth status of the plants [30]. During the photographing process, we take photographs at crucial time points. This includes before the freezing treatment, as well as at specific intervals after recovery from the freezing treatment. By capturing images at these key stages, we can observe any changes in plant height and stem thickness over time. This allows us to assess the recovery rate and cold tolerance of different wheat varieties. For example, we take photographs at 0 d before the freezing treatment, 5 d after recovery, 10 d after recovery, and 18 d after recovery. This provides us with a comprehensive set of data that we can analyze and compare.

By combining regular measurements of plant height and stem thickness with photographic records, we can obtain a comprehensive understanding of the growth status and cold tolerance of wheat varieties. This information is crucial for breeders and farmers, as it helps them select varieties that are suitable for their specific growing conditions and have the potential for high yields. Additionally, by comparing the performance of different varieties, we can gain insights into the genetic factors that contribute to cold tolerance in wheat, which can guide future breeding efforts.

2.2.4 RNA extraction and cDNA library construction

In molecular biology experiments, RNA extraction and purification is a crucial step. To ensure the accuracy and reliability of the experimental results, we followed the detailed instructions of TaKaRa MiniBEST Plant RNA Extraction Kit to extract

RNA samples precisely and without errors [31]. The kit is specifically designed for plant RNA extraction and can efficiently isolate high-quality RNA from plant tissues.

After the extraction was completed, we rigorously tested the integrity and purity of the RNA using the Ultra-Micro Nucleic Acid Protein Assay. This step ensured that the RNA samples we obtained were not contaminated or degraded, providing a solid foundation for subsequent experiments.

Next, to remove genomic DNA from the RNA, we used 5×gDNA Eraser Buffer and gDNA Eraser to construct a 20 μl reverse transcription reaction system. The reaction was carried out at 42 °C for 2 min, which effectively removed the genomic DNA from the RNA sample and avoided its interference with the results of subsequent experiments. When the reaction was completed, we cooled it to 4 °C and added PrimeScript RT Enzyme Mix I, RT Primer Mix*4 and 5×PrimeScript Buffer 2 for amplification, which successfully reverse transcribed the RNA into cDNA, providing high-quality cDNA for subsequent gene expression analysis or other molecular biology experiments library for subsequent gene expression analysis or other molecular biology experiments.

2.2.5 qRT-PCR verification of gene expression

In this study, we explored in depth the mechanisms of gene expression in plants under cold conditions. First, we screened six potential key cold resistance genes from the rich transcriptome database, including three yet-to-be-reported CBF1 genes, two CBF genes, and three ICE1 genes. These genes may play important roles in plant response to low temperature stress.

To verify the cold-resistant functions of these genes, we employed qRT-PCR to determine the expression of these genes under different temperature treatments. In our experiments, we carefully designed specific primers for these genes, which are listed in detail in Table 2.1. In order to accurately quantify gene expression levels, we chose an internal reference gene, β-actin, as a control to correct for quantitative differences in the cDNA used as a template.

Next, we extracted total RNA from the leaves and successfully synthesized first-strand cDNAs using the M-MuLV First Strand cDNA Synthesis Kit. qRT-PCR experiments were subsequently performed using a fluorescent PCR quantifier to accurately measure the expression levels of these genes at different temperatures. In the data analysis stage, we adopted the 2^-ΔΔCT method to calculate the relative gene expression and used GraphPad Prism 9.5.0 software to draw intuitive and easy-to-understand graphs to make the experimental results more clear and concise. [32].

Table 2.1 – **List of cold-resistant gene qRT-PCR primers**

Gene ID	Gene name	Primer sequence	
CS4A02G433200	*CBF1*	GACCAGTACAACTACGGCGG	
		CCAGATGCGGGACTTCTTGT	
CS4A02G433300	*CBF1*	GCAAGAAGTCCCGCATCTGG	
		GAGGAATTCGTCGCGTCTTTG	
CS6A02G256900	*CBF1*	GGAGGTAGCAGTTGCACCAA	
		GTCTCCGACAGTCGCTGAAG	
CSIA02G137500	*CBF*	AAGATGGCAGCCAC GGAC	
		CGGAAGAGGAAGATCCCGTG	
CSID02G133000	*CBF*	GAAGAC GGCAGCCACAGAC	
		CGGAAGAGGAAGATCCCGTG	
CS4A02G263300	*ICE1*	GCGTGAAGGAGGAACTGACA	
		ATCCTTGCACTCGGCCTTG	
CS4D02G051600	*ICE1*	ACATCCGCATGATGTGCTCT	
		GCACTGCTCAGCCTTGAAGA	
CS7A02G311800	*ICE1*	GATGGGGCAGGAGGCTG	
		TTGAGGAGCGGGAACATCTG	

2.2.6 Gene cloning and expression

In an in-depth exploration of the mechanisms of cold resistance in plants, we first searched and obtained the sequence information of two key genes, CBF and ICE1, in the National Center for Biochemistry Information (NCBI) database. In order to study the functions of these genes in plants more precisely, we designed highly specific oligonucleotide primers based on these gene sequences using Primer 5 software (Tab. 2.2). These primers were designed to ensure that the target gene

sequences could be accurately and efficiently identified and amplified during subsequent PCR amplification and gene cloning.

During the process of gene cloning, we added a variety of key enzymes and reagents to the cDNA system. First, DNA polymerase is indispensable to synthesize new DNA strands based on template DNA. Second, we added DNA restriction endonuclease, which recognizes and cuts specific sequences on the DNA strand for subsequent gene recombination and ligation. In addition, T4 DNA ligase was used to join two DNA fragments to form a complete gene sequence. Finally, we added four dNTP (deoxyribonucleotides), which are the basic raw materials for DNA synthesis. [33].

To verify the success of the gene cloning, we used the classic technique of agarose gel electrophoresis. Agarose gel electrophoresis utilizes the molecular sieving action of the agarose gel and the dual effect of electrophoresis to separate DNA fragments according to their size and charge properties. By observing the gel image after electrophoresis, we can clearly see the number and size of DNA fragments, and thus determine whether the gene cloning process is successful or not. This step not only provides an important reference basis for our experiment, but also lays a solid foundation for the subsequent research.

Table 2.2 – **List of primers for gene cloning**

Gene ID	Primer sequence
CS4A02G433200	ATGGACCAGTACAACTACGGCGGCGGC TCAGTAGCTGAGCGCGACGCC
CSIA02G137500	ATGATGCGTAAGAAACTTTCGGGCGG CTACTGGCCATCCTCGGGGAAGCGCGA

Conclusions to chapter 2

1. To further elaborate on our research methodology, Tai 113 and C8178, two distinct varieties of wheat, were chosen as the experimental materials in this study. We employed both hydroponic and soil cultivation methods to cultivate the wheat seeds, aiming to observe and document their growth

patterns under different cultivation conditions. To assess the physiological status of the wheat plants, we utilized various physiological index measurement kits, such as those for chlorophyll content and catalase activity. These measurements allowed us to gain insights into the plant's vitality and metabolic status. Furthermore, phenotypic data measurements, including plant height and stem thickness, were also taken to provide quantitative insights into the growth and development of wheat.

2. As we delve deeper into understanding the cold resistance of wheat, the next phase of our experiments focused on gene expression analysis. Firstly, we extracted RNA from the wheat samples and constructed a cDNA library, which serves as a template for subsequent molecular analysis. This step was crucial in identifying the genes that are actively expressed in response to cold stress. Subsequently, we utilized qRT-PCR, a highly sensitive and quantitative technique, to verify the expression levels of these genes. By analyzing the gene expression patterns, we aimed to gain a comprehensive understanding of the molecular mechanisms underlying wheat's cold resistance. This integrated approach, combining phenotypic and molecular data, will not only enhance our knowledge of wheat cold resistance but also provide valuable insights for the improvement of crop resilience and food security.

CHAPTER 3
EXPERIMENTAL PART

3.1 Cold resistance of different varieties of wheat seeds at germination stage

During our rigorous experiments with hydroponically grown seeds, we delved deep into the nuances of germination and survival under various conditions. Our observations were particularly striking when comparing the two wheat varieties, C8178 and Tai 113. Prior to the application of cold stress, we found that the C8178 seeds exhibited a notably higher relative germination number compared to Tai 113. This early indicator suggested that C8178 might possess some inherent resilience or genetic predisposition towards successful germination. However, the real test came after subjecting the seeds to cold treatment. Here, the differences became even more apparent. The survival rate of C8178 seeds was significantly greater than that of Tai 113 (Tab. 3.1). This result underscores not only the seeds' ability to germinate, but also their robustness in adverse environments.

Intriguingly, we observed that the Tai 113 seeds that failed to germinate successfully displayed clear signs of deterioration. Their endosperm, the nutrient-rich tissue that supports the embryo, was weakened to the point that it even flowed out. This physical degradation indicates a lack of resilience in the Tai 113 variety when confronted with cold stress.

In contrast, the C8178 seeds displayed a marked resilience, even under the same cold treatment conditions. Their ability to germinate and survive in such an environment tentatively suggests that C8178 possesses a stronger performance in terms of cold resistance compared to Tai 113. This finding holds promise for future breeding programs aimed at developing wheat varieties that can withstand harsher environmental conditions.

Table 3.1 – **List of Survival of wheat seeds**

Cultivates	Germination number before cold treatment	Survival rate after cold treatment
Tai 113	42/50	35.7%
	41/50	36.6%
	40/50	35.0%
C8178	45/50	82.0%
	46/50	84.1%
	43/50	80.5%

3.2 Phenotypes of cold resistance of different wheat varieties

In the cold tolerance study targeting wheat seedlings, we paid special attention to the performance of two varieties - Tai 113 and C8178 - under low temperature environment. After a period of low temperature domestication at 4 °C, we further subjected these two groups of wheat to a more extreme cold treatment of -18 °C for 3 h. During the subsequent recovery process, we measured the plant height and stem thickness of the wheat at regular intervals. From the data in Table 3.2, it is obvious that the plant height and stem thickness of C8178 grew more than that of Tai 113 as the number of days of recovery increased, which further confirms that C8178 showed stronger resistance in the face of the cold environment. (Fig. 3.1, Tab. 3.2).

Table 3.2 – **List of Wheat seedling phenotype**

Cultivates	Processing days	Plant heigh	Stem diamet
Tai 113	5d	12.83±0.26	0.17±0.02
	10d	17.70±0.14	0.24±0.06
	18d	27.23±0.22	0.30±0.02
C8178	5d	12.98±0.31	0.19±0.03
	10d	20.07±0.20	0.25±0.01
	18d	32.83±0.09	0.34±0.03

After 30 d of low temperature domestication at 4 °C, the wheat varieties were started to undergo cold treatment at -18 °C. The plant height and stem thickness of

wheat at different times of growth were determined, and the results are listed in Table 3.2. Low temperature stress had a significant effect on the growth of different varieties of wheat [13] (Fig. 3.1), and the plant height and stem thickness of Tai 113 increased to different extents compared with that of C8178. Leaf curling and drooping started to appear after 5 d of treatment in Tai 113, and most of the tender leaves close to the ground began to turn yellow and soft, with poor resistance to stunting, and the yellowed leaves began to dry up at 15 d of treatment, some of which were in a state of basic death. At 18 d after the cold treatment, C8178 was in the best growth condition, with the plant height and stem thickness reaching the maximum values during the observation period. C8178 was not significantly affected by the cold treatment, and the plant was very upright for 18 d after the treatment, and a small amount of yellowing started to appear on the lower leaves close to the soil after 15 d of the treatment. From the comparison of morphological indexes, the expression rate of physiological phenomena produced by cold stress in C8178 after cold treatment was significantly smaller than that of Tai 113, the ability of C8178 to resist abiotic stress was higher than that of Tai 113, which indicated that there was indeed a cold-resistant gene with a high expression level in the C8178 variety.

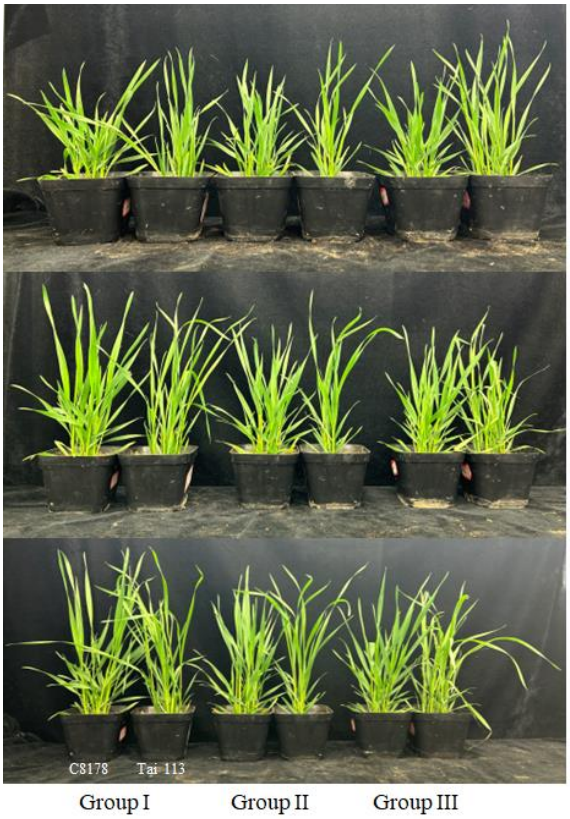


Figure 3.1 – Wheat photos

3.3 Cloning of cold resistance genes

After an in-depth analysis of the RT-PCR results, we revealed significant changes in gene expression in wheat under cold stress at -18 °C not only on a statistical level but also in a biological sense. Through ANOVA (Analysis of Variance) and Significant Difference Analysis (Fig. 3.2), we were supported by conclusive data that provided strong evidence for revealing the molecular mechanisms of cold resistance in wheat. Among the many genes examined, two specific genes, CS4A02G433200 (CBF1) and CSIA02G137500 (CBF), showed particularly significant up-regulation of expression in the C8178 line. This finding not only gave us a clearer understanding of the cold resistance mechanism in wheat, but also made us realize that these two genes may play a central role in the low temperature response of wheat. We hypothesize that these two genes may encode some crucial proteins under low temperature conditions, which significantly enhance the cold resistance of wheat through various pathways such as improving cell membrane stability, enhancing enzyme activity or promoting metabolite synthesis. Meanwhile, we also noticed that the expression of two genes, CS4D02G051600 (ICE1) and CS7A02G311800 (ICE1), although also increased at -18 °C, the increase was smaller compared with the first two genes. This observation led us to speculate that these two genes may play a more indirect or auxiliary role in the cold tolerance response of wheat. Although their effects on cold resistance may not be as significant as those of the first two genes, their expression changes under low-temperature stress still provide us with new perspectives to study the mechanism of cold resistance in wheat. It is noteworthy that among all the genes examined, their expression remained stable under ambient conditions, both in the C8178 line and in the other lines. This finding further confirms that temperature is a key factor influencing gene expression in wheat, and that this effect is realized by regulating the expression of specific genes. This not only provides clues for us to understand how wheat adapts to different temperature environments, but also provides a possible direction for us to improve the cold resistance of wheat by means of genetic engineering. In conclusion, through

in-depth analysis of the RT-PCR results, we not only revealed significant changes in gene expression in wheat under low-temperature stress, but also identified several genes that may play key roles in the cold resistance mechanism of wheat. These findings not only provide a new perspective for us to understand the cold resistance mechanism of wheat, but also provide an important reference for future improvement of cold resistance in wheat by genetic engineering.

For the increased expression of three genes, CSID02G133000 (CBF), CS4A02G433300 (CBF1), and CS4A02G263300 (ICE1), at -18 °C, we further hypothesized that these genes were activated under extreme low-temperature conditions and were involved in the cold-resistance mechanism of wheat. Under low-temperature stress, these genes may enhance the cellular adaptation of wheat by encoding specific proteins to improve its cold tolerance.

At the same time, we also noticed that at 4 °C, the expression of these genes, although decreased, was still higher than the expression level under room temperature conditions. This may imply that these genes are still responsive in the suprazero temperature range, and this responsiveness may be important for the growth and development of wheat in the cold season.

As for the low expression of CS6A02G256900 (CBF1) at -18 °C, we do believe that this may be related to the insufficient freezing treatment time. In future experimental designs, we will consider increasing the freezing treatment time to gain a more comprehensive understanding of the role of this gene in the cold resistance mechanism of wheat. In addition, this finding also reminds us that we need to strictly control the various treatment conditions during the experiment to ensure the accuracy and reliability of the results.

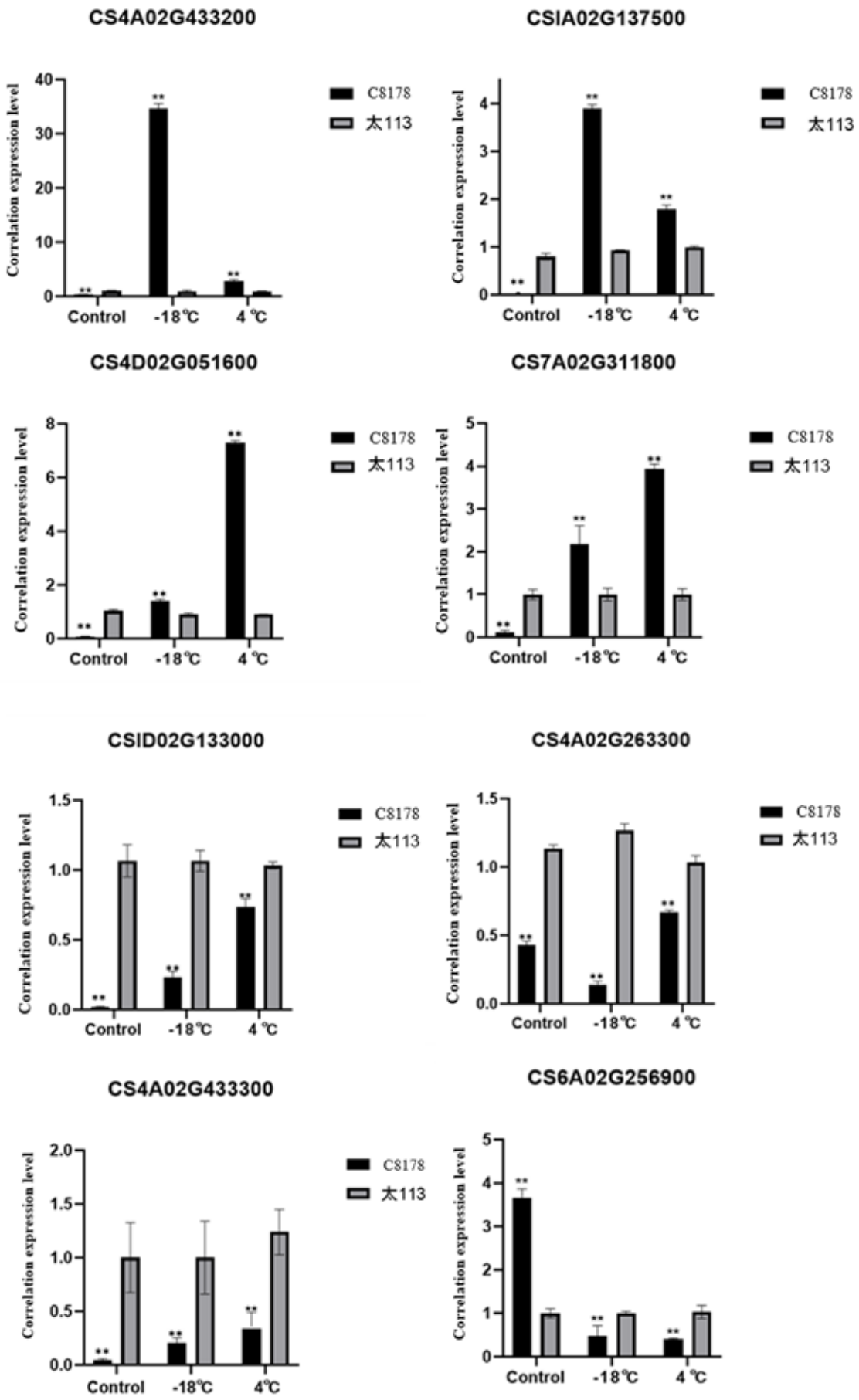


Figure 3.2 - Relative expression of cold resistance genes

3.4 Cloning of cold resistance genes

According to Fig. 3.3, According to the data provided in Figure 3. we can clearly see that the expression of the two genes, CS4A02G433200 and CSIA02G137500, was significantly higher than that at room temperature versus 4 °C in the C8178 variety of wheat after -18 °C cold treatment. This result suggests that these two genes are particularly sensitive and responsive to cold stress in the early stages of cold resistance in wheat. In view of their importance, we decided to perform cloning operation on these two genes in order to further study and explore their roles in the cold resistance mechanism of wheat in depth.

By searching https://plants.ensembl.org/index.html, an authoritative gene database, we successfully obtained the sequence information of these two genes, which provided a solid foundation for the subsequent experiments:

（1） CS4A02G433200：

ATGGACCAGTACAACTACGGCGGCGGCGTGGCCTACTACGGCAGCA
CCACCGGCGGCGTAGGCGACAACGGCCAGGGCGGCGGGTACGCGACGGT
GACGTCGGCGCCGCCGAAGCGGCCGGCGGGGCGGACCAAGTTCCGGGAG
ACGCGCCACCCGGTGTACCGGGGCGTGCGCCGGCGCGGCGCGGCCGGGC
GGTGGGTGTGCGAGGTGCGCGAGCCCAACAAGAAGTCCCGCATCTGGCT
CGGCACCTTCGCCAGCCCCGAGGCCGCGGCCCGCGCCCACGACGTCGCCG
CGCTCGCGCTCCGGGGCCGCGCCGCCTGCCTCAACTTCGCCGACTCGGCC
ACGCTGCTCGCCGTCGACCCCGCCACGCTCCGCACCCCCGACGACATCCG
CGCCGCCGCCATCGCGCTCGCCGAGACCGCGTGCCCCGCCGCGCCCGCGT
CCTCGTCGTCCGTGGCCGCCGCCGTGGCGTCCGCGCCGGCGCCCCCGATG
ACGATGATGCAGTTTGACGACTACGCGATGCAGTACGGCGGCATTGGGG
ACTTGGACCAGCATTCCTACTACTACGACGGGTTGAGCGCCGCCGGTGGC
GACTGGCCGAGCGGCTCGCACATGGACGGAGCCGACGACGACTGCAACG
GCAGCGGTGGCTACGGCGCCGGCGAGGTCGCGCTCTGGAGCTACTGA

（2） CSIA02G137500：

ATGATGCGTAAGAAACTTTCGGGCGGCGGAATGGACGCGACGGTGG
ACGAGCTGAGCGCGGCGTATAAGGAGTTCGTGGCGGCTGCGGTGGCCGT

GATGGAAGCTCGCGAGCAGTCGGGCGGCCAGAAGATGGCAGCCACGGAC
GCGGCGCTTGAAGCCTTCAAGCAGCGATGGGAGCTCTTCCGCGTCTCCTG
CGACCACGCCGAGGAGCTCGTTGAGTCCATCCGCCAGCGCATCGGCTCCG
AGTGTCTCGTCGACGAGGCCACGGGATCTTCCTCTTCCGCCTCCACGCCC
GCAAGCGTCGCGCTGGCTGCCCCCGGCATCAAGCCAATCAGCGCCGTCCG
CCTCGAGCAGATGAGCAAGGCCGTCCGCTGGCTCGTTATCGAGCTTCAGC
ACGGCGTCGGAGGGCCCTCAGCTGCCGGACCTGGCGGTGGCGTCTCAACC
CCGGCTGCCGGCGCCGGAGGGCAGCATGTGCACGGCGGGGTCGAATCGC
GCTTCCCCGAGGATGGCACCCAGTAG

In the process of in-depth research on the mechanism of cold resistance in wheat, we accurately selected the genes CSIA02G137500 and CS4A02G433200 in C8178 cultivar, which had particularly significant changes in expression under cold stress, through analysis of variance (ANOVA) and analysis of significance (ANS). In order to verify whether the two genes were successfully cloned, we used BM2000 DNA marker for agarose gel electrophoresis experiments. Figure 3.3 demonstrates the detailed electrophoresis results, from which it can be clearly seen that the electrophoretic band of the CSIA02G137500 gene corresponds to its expected length of 157 bp, while the electrophoretic band of the CS4A02G433200 gene accurately reflects its length of 237 bp. The clear display of the bands of these two genes not only proves the initial success of the gene cloning experiment, but also lays a solid foundation for our subsequent in-depth study of the functions of these two genes.

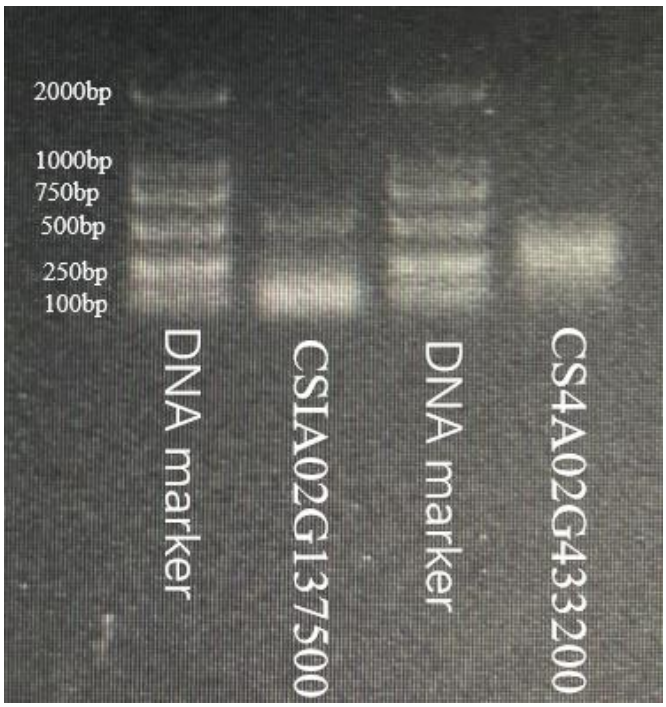


Figure 3.3 - Cloning results

Conclusions to chapter 3

1. The results of a comparative cold treatment experiment with C8178 and Tai 113 wheat varieties were exceptionally interesting. It was not just a simple comparison, but a vivid demonstration that vividly presented C8178's excellence in cold resistance. In the early stages of seed germination, C8178 showed amazing resilience. In both germination and survival rates, C8178 significantly outperformed Tai 113, and under further cold stress conditions, C8178 seedlings showed remarkable resilience. Their leaves were green, straight and strong, and the whole plant grew taller and more vigorous. The superiority of this growth pattern was not only visible to the naked eye, but also verified by dry weight measurements. The higher biomass accumulation of C8178 under low-temperature stress is further evidence of its superiority in cold resistance. This finding, undoubtedly, provides a new direction for the breeding of cold-resistant wheat, and C8178 is undoubtedly a highly promising candidate.

2. Subsequently, we conducted an in-depth analysis of the expression of key cold resistance genes in C8178 and Tai 113, which provided us with more in-depth insights into the molecular mechanisms behind the cold resistance of C8178. Among the many cold resistance genes, CBF1 and ICE1 are undoubtedly two crucial players. In both varieties, the expression levels of CBF1 and ICE1 genes were significantly higher in C8178 than in Tai 113, a finding that makes us wonder about the role of these genes in the cold resistance of C8178. Perhaps, it is the high expression of these genes that conferred stronger cold resistance to C8178. They may promote the production of protective proteins or metabolites by activating downstream signaling pathways, thereby mitigating the damage caused by low temperatures to the plant. In addition, the combined action of multiple cold resistance genes may also have provided C8178 with more comprehensive cold resistance. This discovery not only provides a new perspective for us to understand the mechanism of cold resistance in wheat, but also provides strong support for us to breed new cold-resistant wheat varieties. the excellent cold resistance of C8178 gives us hope to ensure food security under harsh environmental conditions. In the future, we hope to introduce the excellent genes of C8178 into other wheat varieties through breeding technology, and breed more wheat varieties that can thrive in cold environments, thus contributing to global food security.

CONCLUSIONS

1. After cold treatment, we observed that the expression of cold resistance genes in different wheat varieties showed significant changes. This was specifically manifested in the following aspects:

(1) CBF genes: CBF genes play a pivotal role in the plant cold resistance mechanism, and they belong to a class of key cold resistance genes, which encode transcription factors that can effectively regulate a series of genes closely related to cold resistance. In our study, the expression of CBF genes in C8178 wheat showed a significant upward trend after cold treatment. This phenomenon usually started to appear within a few hours after treatment, and the increase in CBF gene expression further triggered a series of physiological and biochemical responses, which helped wheat to better adapt to the low-temperature environment, thus enhancing its cold tolerance.

(2) ICE1 genes (antifreeze protein genes): In addition to the CBF genes, we also paid attention to the changes in the expression of antifreeze protein genes. The expression of these genes is also increased under low temperature induction. In particular, the increased expression of antifreeze protein genes helps to lower the freezing point in the cell, thus effectively preventing the cell from freezing damage. Although the rise in expression of these genes after cold treatment may not be as significant as that of the CBF genes, they also have a non-negligible role in improving cold resistance in wheat. Therefore, when researching and breeding cold-resistant wheat varieties, we should comprehensively consider the changes in the expression of multiple cold-resistant genes in order to more comprehensively evaluate and enhance the cold-resistant performance of wheat.

2. With the increasingly severe global climate change, wheat production is encountering unprecedented challenges, especially the problem of low-temperature cold damage, which has become a difficult problem that cannot be ignored. In this paper, we analyzed the cold resistance of two wheat varieties, Tai 113 and C8178, and successfully cloned the related cold resistance genes, which opened up a new path for the research of cold resistance breeding in wheat. However, this is only the

starting point for exploring the mystery of cold resistance in wheat, and we still need to deepen this research in the future.

3. Looking ahead, it is critical to deepen our understanding of the mechanisms of cold resistance in wheat. At present, we have initially identified some of the genes and proteins associated with cold resistance, but this is only the tip of the iceberg. With advancing biotechnology, especially high-throughput sequencing and proteomics, we can hope to comprehensively and systematically reveal the molecular response mechanisms of wheat under low-temperature stress. This will not only improve our knowledge of wheat's adaptation to low-temperature environments, but also provide a solid theoretical foundation for breeding wheat varieties that are more resistant to cold. Translating theoretical knowledge into practical applications is equally important. Using gene editing techniques such as CRISPR-Cas9, we can precisely modify wheat genes to enhance its cold resistance [34]. This technology offers the possibility of breeding new cold-resistant wheat varieties, but we must be prudent in the application process to ensure that the edited crop is both safe and efficient.

4. In addition to biotechnology, combining traditional breeding with modern methods is crucial. Traditional breeding has yielded excellent wheat varieties, yet there's still room for cold resistance improvement. Integrating biotech tools can enhance precision in selecting high-yield, cold-resistant wheat. Multidisciplinary approaches, especially agronomy and bioinformatics, offer new avenues for wheat research. Bioinformatics can pinpoint key cold-resistance genes. Future studies should integrate theory and practice, deeply explore cold resistance mechanisms, and bravely experiment with new breeding methods, all while considering ecological adaptability and sustainability to ensure food security.

5. Despite the many challenges, research on cold resistance in wheat holds great promise. In this process, we must maintain patience and determination, not be impatient, and work steadily. We hope that through continuous research and practice, we can breed more high-yielding, high-quality and cold-tolerant new wheat varieties to contribute to global food security.

LIST OF REFERENCES

1. Hassan, M.A., et al., *Cold Stress in Wheat: Plant Acclimation Responses and Management Strategies.* Front Plant Sci, 2021. 12: p. 676884.
2. Xiao, L., et al., *Estimating spring frost and its impact on yield across winter wheat in China.* Agricultural and Forest Meteorology, 2018. 260-261: p. 154-164.
3. Aroca, R., R. Porcel, and J.M. Ruiz-Lozano, *Regulation of root water uptake under abiotic stress conditions.* Journal of Experimental Botany, 2012. 63(1): p. 43-57.
4. Zhang, X., et al., *Spatial distribution and temporal variation of the winter wheat late frost disaster in Henan, China.* Acta Meteorologica Sinica, 2011. 25(2): p. 249-259.
5. Wang, X., et al., *Identification of Mild Freezing Shock Response Pathways in Barley Based on Transcriptome Profiling.* Front Plant Sci, 2016. 7: p. 106.
6. Zhao Lishang, Shi. Mei, Chung-Hsun, and Zong-Yao Pan, Research and discussion on frost damage of wheat. Agricultural Science and Technology Newsletter, 2005(10): p. 15-16.
7. Ni Shengli, Zhang Guohong, and Li Xingmao, Overview of wheat cold resistance research. Gansu Agricultural Science and Technology, 2008(08): p. 23-26. 8.
8. Li W G, Impacts of low-temperature cold damage on the growth process of rice and countermeasures. Agricultural Technology Service, 2016. 33(01): p. 67.
9. Duan Longfei, Research on photosynthetic characteristics and cold resistance of Chinese golden leaf elm and its asexual lines. 2015.
10. zhang yongfeng, Wheat cultivation technology. Modern Rural Science and Technology, 2024(03): p. 21.
11. Trnka, M., et al., *Adverse weather conditions for European wheat production will become more frequent with climate change.* Nature Climate Change, 2014. 4(7): p. 637-643.

12. Xie, Fengxian, Wang, Zhiyu, and Zhang, Ziyang, Effects of spring low-temperature stress on fruit set and leaf physiological properties of different wheat varieties. Anhui Agricultural Science, 2018. 46(32): p. 33-35+39.
13. Zhou M., Characterization of wheat cold response based on transcriptomics and metabolomics. 2018.
14. Caccialupi, G., et al., *The Triticeae CBF Gene Cluster—To Frost Resistance and Beyond.* Cells, 2023. 12(22): p. 2606.
15. Peng, P.-H., et al., *Cold response in Phalaenopsis aphrodite and characterization of PaCBF1 and PaICE1.* Plant & cell physiology, 2014. 55 9: p. 1623-35.
16. *Editorial Board and Contents.* Trends in Plant Science, 2018. 23(7): p. i.
17. Shi, Y., et al., *Ethylene Signaling Negatively Regulates Freezing Tolerance by Repressing Expression of CBF and Type-A ARR Genes in Arabidopsis* The Plant Cell, 2012. 24(6): p. 2578-2595.
18. Tondelli, A., et al., *Inside the CBF locus in Poaceae.* Plant Science, 2011. 180(1): p. 39-45.
19. Guo, X., D. Liu, and K. Chong, *Cold signaling in plants: Insights into mechanisms and regulation.* Journal of Integrative Plant Biology, 2018. 60(9): p. 745-756.
20. Guo, J., et al., *Characterization and expression profiling of the ICE-CBF-COR genes in wheat.* PeerJ, 2019. 7: p. e8190.
21. Badawi, M., et al., *Structure and functional analysis of wheat ICE (inducer of CBF expression) genes.* Plant Cell Physiol, 2008. 49(8): p. 1237-49.
22. Crosatti, C., et al., *Genetic analysis of the expression of the cold-regulated gene cor14b: a way toward the identification of components of the cold response signal transduction in Triticeae.* Canadian Journal of Botany, 2003. 81(12): p. 1162-1167.
23. Zhang, X., Research on image-based methods for counting wheat ears and spikelets. 2021.

24. niu yuqi, et al., High-yielding wheat variety tai113 and supporting cultivation technology. China Seed Industry, 2017(11): p. 71-72.
25. Tou Y., et al., Research progress on the application of hydroponic wheat seedlings in livestock production. Journal of Animal Nutrition, 2023. 35(07): p. 4216-4223.
26. Zhang Huiping, et al., Effects of different potassium fertilizers on the hydroponic effect of wheat seeds. Humic acid, 2024(01): p. 45-49. 27.
27. Yang Jiayu, et al., Effects of deep tillage-rotary tillage and organic fertilizer on soil physical properties in black soil farmland. Soil Bulletin, 2021. 52(06): p. 1290-1298. 28.
28. Wu, H. T., et al., Effects of machine sowing depth, seeding rate and fertilizer application on buckwheat inversion and yield. Journal of Grass Industry, 2020. 29(12): p. 61-72.
29. Wang Caixia, Wheat cultivation influencing factors and application strategies of cultivation technology. Grain Science and Economy, 2020. 45(12): p. 109-110. 30.
30. A simple method for photographing young wheat ears. Shanghai Agricultural Science and Technology, 1977(05): p. 16.
31. Guo, M., et al., *First Report of Leek Yellow Stripe Virus Infecting Allium cepa in China.* Plant Disease, 2024. 108(3).
32. Ding, M., et al., *Transcriptome analysis of brassinolide under low temperature stress in winter wheat.* AoB Plants, 2023. 15(2): p. plad005.
33. Zhang, J., et al., *Gene cloning, expression, and characterization of two endo-xylanases from Bacillus velezensis and Streptomyces rochei, and their application in xylooligosaccharide production.* Front Microbiol, 2023. 14: p. 1292726.
34. Zhang, Shujuan, et al., Progress of research on spring cold resistance of wheat. Shandong Agricultural Science, 2017. 49(06): p. 157-162.

APPENDIX

A.

Data from qRT-PCR

4℃-C8178-24h	4℃-Tai 113-24h	-18℃-C8178-3h	-18℃-Tai 113-3h	Control-Tai 113	Control-C8178
31.935	28.287	30.247	27.753	27.498	31.327
32.128	29.988	31.250	27.843	28.239	30.911
31.281	28.503	30.733	28.608	28.155	31.265

4℃-C8178-24h	4℃-Tai 113-24h	-18℃-C8178-3h	-18℃-Tai 113-3h	Control-Tai 113	Control-C8178
32.327	33.494	28.883	32.797	31.954	33.765
33.248	32.700	29.475	32.816	32.314	32.323
32.251	31.890	28.844	33.551	31.903	32.687

4℃-C8178-24h	4℃-Tai 113-24h	-18℃-C8178-3h	-18℃-Tai 113-3h	Control-Tai 113	Control-C8178
36.702	34.128	32.137	30.456	32.288	29.247
36.556	34.072	32.386	30.389	31.589	29.548
37.875	33.654	31.525	30.381	32.394	29.988

4℃-C8178-24h	4℃-Tai 113-24h	-18℃-C8178-3h	-18℃-Tai 113-3h	Control-Tai 113	Control-C8178
28.563	28.078	26.111	28.636	26.960	31.965
28.937	28.156	27.096	28.086	26.300	32.413
28.690	27.629	26.782	27.582	27.042	32.516

4℃-C8178-24h	4℃-Tai 113-24h	-18℃-C8178-3h	-18℃-Tai 113-3h	Control-Tai 113	Control-C8178
31.733	29.659	29.018	27.487	26.173	30.339
31.564	29.887	28.999	26.822	25.744	30.002
31.180	28.689	29.212	26.314	25.353	31.350

4℃-C8178-24h	4℃-Tai 113-24h	-18℃-C8178-3h	-18℃-Tai 113-3h	Control-Tai 113	Control-C8178
35.395	33.379	32.561	29.251	31.180	30.699
35.180	32.063	32.617	29.585	30.634	30.861
34.506	32.481	33.268	30.883	31.339	30.578

4℃-C8178-24h	4℃-Tai 113-24h	-18℃-C8178-3h	-18℃-Tai 113-3h	Control-Tai 113	Control-C8178
26.499	27.917	24.044	23.828	29.376	30.124
27.484	27.195	24.299	23.570	29.580	31.755
26.168	27.766	23.957	22.072	28.095	31.059

4℃-C8178-24h	4℃-Tai 113-24h	-18℃-C8178-3h	-18℃-Tai 113-3h	Control-Tai 113	Control-C8178
33.945	34.588	32.075	33.004	32.285	34.437
34.690	34.347	31.978	32.933	31.630	34.533
33.913	34.138	32.372	32.542	32.086	33.987

4℃-C8178-24h	4℃-Tai 113-24h	-18℃-C8178-3h	-18℃-Tai 113-3h	Control-Tai 113	Control-C8178
20.619	19.216	18.889	18.972	18.994	17.680
20.365	18.935	19.381	18.792	19.049	17.856
20.659	19.063	19.553	18.789	18.824	18.021

4℃-C8178-24h	4℃-Tai 113-24h	-18℃-C8178-3h	-18℃-Tai 113-3h	Control-Tai 113	Control-C8178
25.493	24.993	24.606	23.992	25.416	24.070
25.077	24.984	25.410	23.806	25.332	24.256
25.565	25.189	24.735	23.884	25.227	24.100

B . MANUAL

Code No. RR047A

研究用

TaKaRa

PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time)

说明书

v201905Da

目　　录

● 制品说明

为了准确地进行基因表达量分析，必须满足只有 cDNA 作为模板检出的先决条件，但 Total RNA 中常常混有基因组 DNA，并可以直接作为 PCR 反应的模板进行扩增，因此会造成解析结果不准确。为了避免这种情况发生，通常将检测用引物设计在内含子前后的外显子上，使基因组 DNA 得不到扩增。但是，此方法不适合具有单个外显子的基因或两个外显子之间所跨的内含子过小的基因，同时当基因组上有伪基因存在时、或设计引物对基因组有非特异性扩增时、以及基因信息没被完全解析的生物种等也同样不适合于本方法。在这种情况下，我们常常需要对 Total RNA 样品进行 DNase I 处理，以除去残存的基因组 DNA。而 DNase I 处理通常要进行复杂的纯化操作，同时会造成 RNA 的降解和损失。

PrimeScript RT reagent Kit with gDNA Eraser 是可以除去基因组 DNA 进行 Real Time RT-PCR 反应的专用反转录试剂。Kit 中使用了具有较强 DNA 分解活性的 gDNA Eraser，通过 42℃，2 min 即可除去基因组 DNA。同时由于反转录试剂中含有抑制 DNA 分解酶活性的组分，经过 gDNA Eraser 处理后的样品可以直接进行 15 min 的反转录反应合成 cDNA，因此，20 min 内即可迅速完成从基因组 DNA 去除到 cDNA 合成的全过程。

使用本制品合成的cDNA适用于嵌合法和探针法qPCR分析，可以根据实验目的，选择与TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Code No. RR820Q/A/B)*、TB Green *Premix Ex Taq* (Tli RNaseH Plus) (Code No. RR420A/B)*、Probe qPCR Mix (Code No. RR391S/A/B) 组合使用。

＊ 现已将 Takara Bio 的嵌合法 real-time PCR (qPCR) 产品名称变更为“TB Green series”。
本次只变更产品名称，产品Code和产品性能不受变更影响，可以与以往产品同样使用。

● 制品内容（20 μl 反应×100 次）

1. gDNA Eraser	100 μl
2. 5×gDNA Eraser Buffer*1	200 μl
3. PrimeScript RT Enzyme Mix I*2	100 μl
4. 5×PrimeScript Buffer 2 (for Real Time) *3	400 μl
5. RT Primer Mix*4	400 μl
6. RNase Free dH2O	1 ml×2
7. EASY Dilution (for Real Time PCR) *5	1 ml

*1: 5×gDNA Eraser Buffer 在反转录反应前使用，请务必进行基因组 DNA 的除去反应。

*2: 含有 RNase Inhibitor。

*3: 含有 dNTP Mixture。

*4: 含有 Oligo dT Primer 和 Random 6 mers。

*5: 制作标准曲线时梯度稀释 cDNA 或 RNA 标准品的稀释液。模板 DNA 或 RNA 如果用水或 TE Buffer 稀释时，由于受 Microtube 吸附作用等的影响，往往不能准确地进行稀释，导致实验结果准确度降低。使用本制品时，即使稀释至低浓度也能够进行准确地稀释，容易在宽广范围内获得准确定量的标准曲线。本制品不影响反转录和 PCR 反应，用其稀释后的样品可直接使用。EASY Dilution (for Real Time PCR) (Code No. 9160/9160Q) 也可以单独购买。

注意: EASY Dilution (for Real Time PCR) 请与本公司 Real Time PCR 试剂组合使用，对于其他公司的同类制品的适用性本公司尚未进行确认。

● 试剂盒外必备材料

热循环仪（或 37℃水浴，42℃水浴和 85℃加热块）
反转录反应所用 0.2 ml 和 1.5 ml 的微量反应管
微量移液器和枪头（高压灭菌）

● 保　存: −20℃。

● 特　长

1. 含有去除基因组 DNA 的 gDNA Eraser，只需 2 min 即可除去基因组 DNA。
2. 只需 15 min 即可高效合成 Real Time PCR 反应用模板 cDNA，是进行 2 Step Real Time RT-PCR 反应的理想试剂。
3. 反转录引物使用了 Random 6 mers 和 Oligo dT Primer 混合的 RT Primer Mix，可以均匀合成样品中的各种 cDNA。
4. 本制品提供了 TB Green qPCR 分析法和探针 qPCR 分析法各自适合的反应体系，可以根据分析方法选择体系。
   TB Green qPCR 分析法和探针 qPCR 分析法区别如下：
   · 反转录反应中 RT Primer Mix 的用量。
   · 反转录反应中总 RNA 的用量。
5. Real Time RT PCR 定量需要建立标准曲线，建立标准曲线的条件就是需要将总 RNA 和反转录 cDNA 稀释到较低的浓度。如果用水或 TE Buffer 稀释时，由于模板浓度低不稳定，因而会缩小曲线范围，结果准确度降低。本制品中附加了标准曲线制作用稀释液 EASY Dilution（for Real Time PCR），将 Total RNA 或 cDNA 稀释至低浓度时也能够进行准确稀释，容易在宽广范围内获得准确定量的标准曲线。

● 使用注意

以下为使用本试剂盒时的注意事项，使用前一定认真阅读。

1. 使用本制品合成的 cDNA 与 TB Green 关联制品组合使用时，建议使用：
   TB Green *Premix Ex Taq* II（Tli RNaseH Plus）(Code No. RR820Q/A/B)
   TB Green *Premix Ex Taq*（Tli RNaseH Plus）(Code No. RR420Q/A/B)
   以上制品与本制品组合使用，可以得到可信度高的结果。
   本制品与 TB Green Fast qPCR Mix（Code No. RR430S/A/B）组合使用时，有时反应性能不好，不推荐使用。
2. 当同时需要进行数次反应时，应先配制各种试剂的混合液（Master Mix；其中包括 RNase Free dH_2O、Buffer、酶等），然后再分装到每个反应管中。这样可使所取的试剂体积更准确，减少试剂损失，避免重复分取同一试剂。同时也可以减少实验操作或实验之间产生的误差。
3. gDNA Eraser 和 PrimeScript RT Enzyme Mix I 在使用前要小心地离心收集到反应管底部。由于酶保存液中含有 50%的甘油，粘度高，分取时应慢慢吸取。同时，要使用精确、量程适合的移液枪，并且不要使 Tip 插入液面过深，否则会因 Tip 壁粘着造成损失，而使酶量不足。
4. 5×gDNA Eraser Buffer 和 5×PrimeScript Buffer 2（for Real Time）在使用前需 Vortex 振荡混匀，轻轻离心后使用。
5. 分装试剂时务必使用新的枪头（Tip），以防止样品间污染。

● 操作方法

1. 去除基因组 DNA 反应

按如下成分于冰上配制反应混合液，为了保证反应液配制的准确性，进行各项反应时，应先按反应数+2 的量配制 Master Mix，然后再分装到每个反应管中，最后加入 RNA 样品。

试剂	使用量
5×gDNA Eraser Buffer	2.0 μl
gDNA Eraser	1.0 μl
Total RNA	*1
RNase Free dH_2O	up to 10 μl

↓

42℃　2 min（或者室温 5 min*2）
4℃

*1: 20 μl 反转录反应体系中，TB Green qPCR 法最多可使用 1 μg 的 Total RNA，探针 qPCR 分析法 最多可使用 2 μg 的 Total RNA。
*2: 室温反应时，可以延长至 30 分钟。

2. 反转录反应

反应液配制请在冰上进行。为了保证反应液配制的准确性，进行各项反应时，应先按反应数+2 的量配制 Master Mix，然后再分装 10 μl 到每个反应管中*3。轻柔混匀后立即进行反转录反应。

<TB Green qPCR 法>

试剂	使用量
步骤 1 的反应液	10.0 μl
PrimeScript RT Enzyme Mix I	1.0 μl
RT Primer Mix *4	1.0 μl
5×PrimeScript Buffer 2（for Real Time）	4.0 μl
RNase Free dH2O	4.0 μl
Total	20 μl*5

Master Mix 10 μl

↓

37℃　15 min*6
85℃　5 sec
4℃*7

< 探针 qPCR 分析法>

试剂	使用量
步骤 1 的反应液	10.0 μl
PrimeScript RT Enzyme Mix I	1.0 μl
RT Primer Mix *4	4.0 μl
5×PrimeScript Buffer 2（for Real Time）	4.0 μl
RNase Free dH2O	1.0 μl
Total	20 μl*5

Master Mix 10 μl

↓

37℃　15 min*6
85℃　5 sec
4℃*7

*3: 若不配制 Master Mix，向步骤 1 的反应液中添加试剂时，要先加入 RNase Free dH2O 和 5×PrimeScript Buffer 2 (for Real Time)混合均匀，以使 gDNA Eraser 的活性充分受到抑制， 再添加 RT Primer Mix、PrimeScript RT Enzyme Mix I，轻轻混匀进行反转录反应。
*4: 使用 RT Primer Mix 可以高效合成 cDNA。因为实验目的不同，也可以不使用 RT Primer Mix，而选择 Oligo dT Primer 或 Gene Specific Primer 进行反转录反应，引物使用量如下：
Oligo dT Primer 50 pmol / 20 μl 反应体系
Gene Specific Primer 5 pmol / 20 μl 反应体系
*5: 反转录体系可以根据需要相应扩大。
*6: 使用 Gene Specific Primer 时，建议反转录反应条件设置为 42℃ 15 min。PCR 反应有非特异性扩增时，将温度升到 50℃会有所改善。

*7：合成的 cDNA 需要长期保存时，请于−20℃或更低温度保存。

注意： 1) 在反转录反应中，TB Green qPCR 法的 RT Primer Mix 用量为 1 μl， 探针 qPCR 分析法的用量为 4 μl。

2) 得到的 RT 反应液加入到下一步的 Real Time PCR 反应体系中，其加入量不要超过 Real Time PCR 反应体积的 1/10（V/V）量。

● Real Time PCR

以下是使用本制品进行反转录反应后，选择 TB Green *Premix Ex Taq* II（Tli RNaseH Plus）（Code No. RR820A/B）进行 Real Time PCR 反应的操作方法。

◆应用 Thermal Cycler Dice™ Real Time System 扩增仪的操作方法

1. 按下列组份配制 PCR 反应液（反应液配制请在冰上进行）。

试剂	使用量	终浓度
TB Green *Premix Ex Taq* II (Tli RNaseH Plus)（2×）	12.5 μl	1×
PCR Forward Primer（10 μM）	1.0 μl	0.4 μM*1
PCR Reverse Primer（10 μM）	1.0 μl	0.4 μM*1
RT 反应液（cDNA 溶液）	2 μl*2	
灭菌水	8.5 μl	
Total	25 μl	

*1 通常引物终浓度为 0.4 μM 可以得到较好结果。反应性能较差时，可以在 0.2～1.0 μM 范围内调整引物浓度。

*2 建议在 25 μl 反应液中使用相当于 10 pg～100 ng Total RNA 量的 cDNA 为模板。反转录反应液的加入量不能超过 PCR 反应液总体积的 10%。

2. 进行 Real Time PCR 反应。

建议采用下列图表显示的两步法 PCR 反应程序。如果该程序得不到良好的实验结果时，再进行 PCR 条件的优化。当使用 Tm 值较低的引物或两步法 PCR 反应扩增性能较差时，可以尝试进行三步法 PCR 扩增反应。

两步法 PCR 扩增标准程序：

Stage 1：预变性
Repeat：1
95℃ 30 秒

Stage 2：PCR 反应
Repeat：40
95℃ 5 秒
60℃ 30−60 秒

Stage 3：Dissociation

◆特别提示：

本制品中使用的 *TaKaRa Ex Taq* HS 是利用抗 *Taq* 抗体的 Hot Start 用 DNA 聚合酶，在 PCR 反应前进行模板的预变性，通常设定为 95℃、30 秒。如果高温处理时间过长，会使酶的活性下降，其 PCR 的扩增效率、定量准确度等都会受到影响。

3. 实验结果分析

反应结束后确认 Real Time PCR 的扩增曲线和融解曲线，进行 PCR 定量时制作标准曲线等。分析方法参见仪器的操作手册。

◆ **应用 Applied Biosystems 7300/7500 Real Time PCR System 的操作方法**

按仪器使用说明书要求进行实验操作。

1. 按下列组份配制 PCR 反应液（反应液配制请在冰上进行）。

试剂	使用量	使用量	终浓度
TB Green *Premix Ex Taq* II(Tli RNaseH Plus)（2×）	10 μl	25 μl	1×
PCR Forward Primer（10 μM）	0.8 μl	2 μl	0.4 μM*1
PCR Reverse Primer（10 μM）	0.8 μl	2 μl	0.4 μM*1
ROX Reference Dye or Dye II（50×）*3	0.4 μl	1 μl	
RT 反应液（cDNA 溶液）	2 μl	4 μl	*2
灭菌水	6 μl	16 μl	
Total	20 μl*4	50 μl*4	

*1 通常引物终浓度为 0.4 μM 可以得到较好结果。反应性能较差时，可以在 0.2～1.0 μM 范围内调整引物浓度。

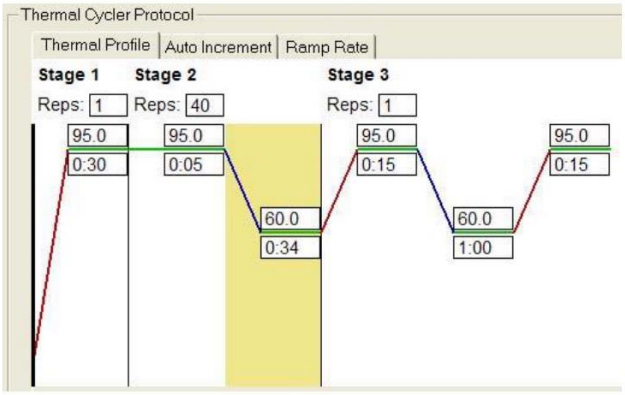
*2 建议在 20 μl 反应液中使用相当于 10 pg～100 ng Total RNA 量的 cDNA 为模板。反转录反应液的加入量不能超过 PCR 反应液总体积的 10%。

*3 ROX Reference Dye II（50×）比 ROX Reference Dye（50×）浓度低，使用 7500 Real Time PCR System 时，请使用 ROX Reference Dye II（50×）。使用 7300 Real Time PCR System 时，请使用 ROX Reference Dye（50×）。

*4 按不同仪器的要求确定反应液的体积。

2. 进行 Real Time PCR 反应。

建议采用下列图表显示的两步法 PCR 反应程序，如果该程序得不到良好的实验结果时，再进行 PCR 条件的优化。若使用 Tm 值较低的引物或两步法 PCR 反应扩增性能较差时，可以尝试进行三步法 PCR 扩增反应。



两步法 PCR 扩增标准程序：

Stage 1：预变性

Reps：1

95℃ 30 秒

Stage 2：PCR 反应

Reps：40

95℃ 5 秒

60℃ 31 或 34 秒*

Dissociation Stage

* 使用 7300 时请设定在 31 秒。
使用 7500 时请设定在 34 秒。

◆特别提示：

本制品中使用的 *TaKaRa Ex Taq* HS 是利用抗 *Taq* 抗体的 Hot Start 用 DNA 聚合酶，如果在 PCR 反应前进行模板的预变性，通常设定为 95℃、30 秒。如果高温处理时间过长，会使酶的活性下降，其 PCR 的扩增效率、定量准确度等都会受到影响。

3. 实验结果分析。

反应结束后确认 Real Time PCR 的扩增曲线和融解曲线，进行 PCR 定量时制作标准曲线等。

● 实验例

A. Total RNA 中混有基因组 DNA 的去除效果验证

[方法]

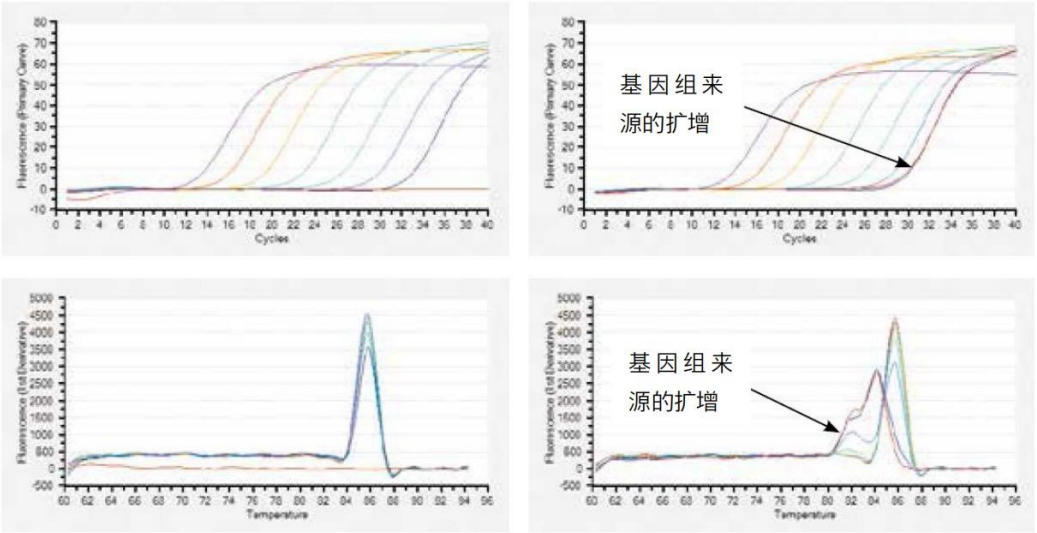
Total RNA 中混入一定量的基因组 DNA 作为模板，使用本制品（Code No. RR047A）与 PrimeScript RT Master Mix (Perfect Real Time)（Code No. RR036A），分别按照各自制品推荐的条件进行反转录反应后，再进行 qPCR 反应，对其结果进行比较。

模　板:	HL60 细胞来源的 Total RNA （0，1 pg，10 pg，100 pg，1 ng，10 ng，100 ng，1 μg） + Human genomic DNA 1 ng
qPCR:	TB Green *Premix Ex Taq* II（TIi RNaseH Plus）(Code No. RR820A)
qPCR 的模板:	反转录反应液各 2 μl
目的基因:	*RPLP1*
Primer:	利用 Perfect Real Time 系统设计 （内参大小：129 bp）
qPCR 装置:	Thermal Cycler Dice Real Time System // (Code No. TP900)

[结果]

即使含有基因组 DNA 的 Total RNA，使用 RR047A 中含有的 gDNA Eraser 处理，可以完全去除基因组 DNA，只能得到 cDNA 来源的扩增产物。

RR047A　　RR036A



B. cDNA 合成效率的比较

[方法]

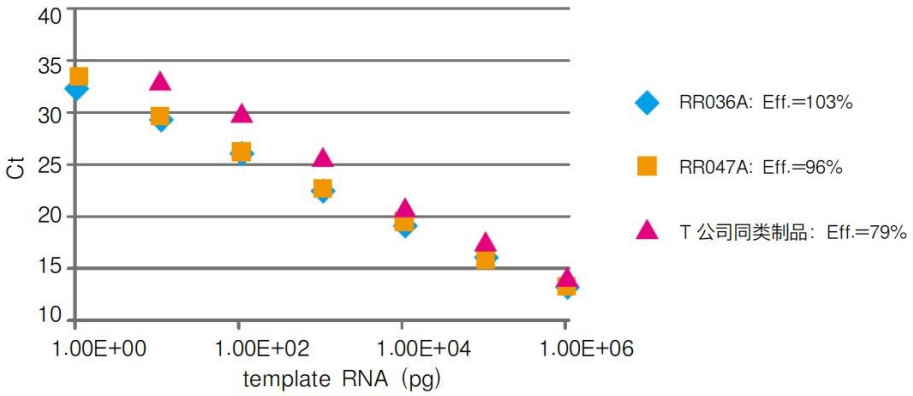
使用梯度稀释的 Total RNA 作为模板，使用本制品（Code No. RR047A）、T 公司同类型制品以及 PrimeScript RT Master Mix (Perfect Real Time)（Code No. RR036A），分别按照各自制品推荐的条件进行反转录反应、qPCR 反应，将结果进行比较。

模　板:	HL60 细胞来源的 Total RNA （0，1 pg，10 pg，100 pg，1 ng，10 ng，100 ng，1 μg）
qPCR:	TB Green *Premix Ex Taq* II（TIi RNaseH Plus）(Code No. RR820A)

qPCR 的模板：　反转录反应液各 2 μl
目的基因：　*RPLP1*
Primer:　利用 Perfect Real Time 系统设计
qPCR 装置：　Thermal Cycler Dice Real Time System //（Code No. TP900）

[结果]

RR047A 的 cDNA 合成效率与不进行基因组 DNA 去除的 RR036A 的效率相当，比 T 公司同类型制品的 cDNA 合成效率高。另外，RR047A 比 T 公司制品反应时间短。



	RR047	T公司同类制品
DNA去除反应	42℃　2分钟	37℃　5分钟
反转录反应	37℃　15 分钟	37℃　15 分钟 50℃　5 分钟
	85℃　5 秒	98℃　5 分钟
反应时间	约 17 分钟	约 30 分钟

本公司比较结果

● 附　录

RNA 样品制备

本制品是将 RNA 反转录成 cDNA 的专用试剂。RNA 的纯度会影响 cDNA 的合成量，而制备 RNA 的关键是要抑制细胞中的 RNA 分解酶和防止所用器具及试剂中的 RNA 分解酶的污染。因此，在实验中必须采取以下措施：戴一次性干净手套；使用 RNA 操作专用实验台；在操作过程中避免讲话等等。通过以上办法可以防止实验者的汗液、唾液中的 RNA 分解酶的污染。

【使用器具】

尽量使用一次性塑料器皿，若用玻璃器皿，应在使用前按下列方法进行处理。

（1）干热灭菌（180℃，60 min）

（2）用 0.1% DEPC（焦碳酸二乙酯）水溶液在 37℃下处理 12 小时。然后在 120℃下高压灭菌 30 分钟以除去残留的 DEPC。

RNA 实验用的器具和仪器建议专门使用，不要用于其它实验。

【试剂配制】

用于 RNA 实验的试剂，需使用干热灭菌（180℃，60 min）或用上述方法进行 DEPC 水处理灭菌后的玻璃容器盛装（也可使用 RNA 实验用的一次性塑料容器），使用的无菌水需用 0.1%的 DEPC 处理后进行高温高压灭菌。

RNA 实验用的试剂和无菌水都应专用，避免混用后交叉污染。

【制备方法】
使用简单的RNA纯化方法即可获得满足于RT-PCR反应的RNA(只需少量的RNA便可进行RT-PCR反应)。但为了保证实验的成功率，建议使用GTC法（异硫氰酸胍法）制备的高纯度RNA。
从培养细胞、组织中提取时，使用RNAiso Plus（Code No. 9108/9109)。纯化的RNA用灭菌水或灭菌的TE缓冲液溶解。

● 关联产品

PrimeScript™ RT reagent Kit (Perfect Real Time) (Code No. RR037Q/A/B)
PrimeScript™ RT Master Mix (Perfect Real Time) (Code No. RR036Q/A/B)
TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Code No. RR820Q/A/B)
TB Green® *Premix Ex Taq*™ (Tli RNaseH Plus) (Code No. RR420Q/A/B)
Probe qPCR Mix（Code No. RR391S/A/B)
EASY Dilution (for Real Time PCR) (Code No. 9160/9160Q)
Thermal Cycler Dice™ Real Time System III (Code No. TP950/TP970/TP980/TP990)
Thermal Cycler Dice™ Real Time System II (Code No. TP900/TP960)
Thermal Cycler Dice™ Real Time System *Lite* (Code No. TP700/TP760)
RNAiso Plus (Code No. 9108/9109)

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