TRANSCRIPTOME ANALYSIS OF RICE SEEDLING UNDER COLD STRESS USING RNA SEQUENCING

BY

MISS PATCHARAPORN SUMMAT

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY (BIOTECHNOLOGY)
DEPARTMENT OF BIOTECHNOLOGY
FACULTY OF SCIENCE AND TECHNOLOGY
THAMMASAT UNIVERSITY
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DISSERTATION

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ENTITLED

TRANSCRIPTOME ANALYSIS OF RICE SEEDLING UNDER COLD STRESS USING RNA SEQUENCING

was approved as partial fulfillment of the requirements for
the degree of Doctor of Philosophy program (Biotechnology)

on October 16, 2023

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ABSTRACT

Rice yields are threatened by climate change, particularly cold stress during the seedling stage. This study aimed to identify genes contributing to cold tolerance in rice seedlings. Using RNA sequencing, we identified cold-responsive genes in cold-tolerant and cold-sensitive varieties at the seedling stage after cold stress at 6-8 °C for 24 hours. This period provides different leaf rolling and withering of distinct seedling varieties. A total of 748 differentially expressed genes (DEGs) unique to B30 were identified, with a predominance of upregulated transcription factors (*bZIP*, *bHLH*, *WRKY*, *NAC*, and *AP2/EREBP*). 6 DEGs (*OsFH10*, *ONAC045*, *SnRK2*, *OsISA1*, *OsFER1*, and *OsProT*) exhibited distinct expression patterns in cold-tolerant varieties compared to cold-sensitive ones using RT-PCR. We further investigated their expression levels, methylation status, alternative splicing, and promoter sequences, in cold-stressed seedlings. Notably, all six genes displayed various transcript isoforms in cold-tolerant varieties under cold stress, due to intron retention. Promoter sequence analysis revealed single nucleotide polymorphisms (SNPs) or insertions/deletions (InDels) in *SnRK2*, *ONAC045*, and *OsProT* that differentiated cold-tolerant from cold-sensitive varieties. These polymorphisms could influence promoter activity and gene expression. Subsequently, *ONAC045* SNP and *OsProT* InDel markers were developed and validated using 159 rice germplasm lines for cold tolerance prediction. The

displayed lower sensitivity in cold-tolerant *indica* rice. Conversely, the *OsProT* marker offered slightly better sensitivity for *indica* rice. Both markers exhibited widespread distribution across rice subspecies. Our findings elucidate the cold response mechanisms of these genes and provide insights into their potential application in developing cold-tolerant rice varieties for future agricultural breeding.

Keywords: Cold tolerance, RNA sequencing, Abiotic stress-responsive genes, *OsFH10*, *ONAC045*, *SnRK2*, *OsISA1*, *OsFER1*, *OsProT*, Alternative splicing, DNA methylation, Molecular markers, Promoters

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Patcharaporn Summat

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
A	Adenine base
AS	Alternative splicing
B primer	Bisulfite primer
B30	IR14632-2-3
C	Cytosine base
CT	Control
CS	Cold stress
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
DPY	Dawk Pah Yawm
FDR	Franklin Delano Roosevelt
G	Guanine base
GO	Gene ontology
IR	Intron retention
InDel	Insertion-deletion
Inr	Initiator region
MSA	Marker-assisted selection
MSP	Methylation-specific PCR
M primer	Methylated primer
mRNA	Messenger ribonucleic acid
NB	Nipponbare
PCR	Polymerase chain reaction
QC	Quality Control
QTL	Quantitative trait locus
RNA	Ribonucleic acid
RD31	Pathumthani 80
RT-PCR	Real Time PCR
RPKM	Reads Per Kilobase per Million reads
SNP	Single nucleotide polymorphism
SPR90	Suphan Buri 90

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
T	Thymine base
TBE	Tris-borate-EDTA
TBP	TATA-binding protein
TF	Transcription factors
TSS	Transcription start site
U primer	Unmethylated primer
UTR	5' untranslated region
V	Volt

CHAPTER 1
INTRODUCTION

1.1 INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most crucial cereals in the world, and about half of the world's population consumes rice as a staple food (Chen & Zhao, 2023). Today, rice is cultivated predominantly in Asian countries such as China, India, Japan, Southeast Asia, and Korea (Muthayya et al., 2014). Asia rice grows in a wide range of environments, from high mountainous areas to low-land delta areas (Dunna & Roy, 2013).

Rice, especially tropical *indica* varieties, exhibits high sensitivity to low temperatures (Yan et al., 2023). This susceptibility to cold stress disrupts key cellular processes, leading to a cascade of detrimental effects on plant growth and yield (Ye et al., 2009). Cold stress disrupts fundamental cellular processes, impacting organelles, membranes, and energy production through the electron transport chain. These disruptions lead to a cascade of negative effects, including increased levels of reactive oxygen species (ROS) and electrolyte leakage (EL) (Shahandashti & Amiri, 2018; Yadav, 2010) alongside reduced rates of photosynthesis and metabolism (Yadav, 2010). These cellular stresses manifest as visible symptoms like chlorosis, stunting, and sterility, ultimately compromising grain yield (Yadav, 2010). Rice seedlings are particularly vulnerable to cold stress, experiencing hindered growth, uneven establishment, and weakened development, all of which contribute to subsequent yield reductions (Dong et al., 2019; Ye et al., 2009).

Transcriptome analyses, reflecting gene activity, offer insights into gene structure, regulation, function, and dynamics (Dong & Chen, 2013). RNA sequencing is a powerful deep-sequencing technique for profiling transcriptomes (Wang et al., 2009). Several studies have used transcriptional profiles to identify genes and potential mechanisms underlying cold tolerance responses in rice seedlings (Do Amaral et al., 2016; Shen et al., 2014; Yan et al., 2023; Yang et al., 2015). Notably, cold stress reduces the function of genes related to photosynthesis (Shen et al., 2014), while plastids,

ribosomes, and chloroplasts exhibit pronounced responses (Do Amaral et al., 2016). It also significantly affects genes related to biotic stress defense, cell death, antioxidant systems, phytohormonal signaling, and the metabolism of starch and sucrose (Yan et al., 2023; Yang et al., 2015).

Unveiling the genes underlying plant adaptation and tolerance to cold stress is crucial for elucidating the mechanisms that endow resilience and enable the development of cold-tolerant cultivars. This involves understanding stress signal perception, signal transduction, TF activation, and cold-responsive gene expression (Zhang et al., 2012). Plants' responses to cold stress involve complex stress response pathways and multifaceted gene interactions (Guan et al., 2019; Guo et al., 2019). Notably, cold stress stimulates the high induction of specific TF families like *NAC*, *WRKY*, and *AP2/EREBP* in the cold-tolerant TNG67 seedling (Yang et al., 2015). Additionally, Yan et al., (2023) demonstrated that diverse TF families, including *WRKY*, *NAC*, *bHLH*, *ERF*, *bZIP*, *MYB*, *C2H2*, and *GRAS*, influence cold tolerance in rice seedlings.

Epigenetics, which regulates alterations in gene expression at the transcriptional level without changes in DNA sequence (Barozai & Aziz, 2018; Kumar, 2018; Yaish, 2013), plays a crucial role in plant adaptation to diverse environments (Çelik et al., 2019; Kumar, 2018). DNA methylation, a key epigenetic mechanism, regulates gene expression in response to stress (Lim et al., 2019; Pan et al., 2011). Elucidating CpG methylation patterns in cold-tolerant genes is crucial for understanding gene regulation in cold stress adaptation.

Alternative splicing (AS), a post-transcriptional regulator, diversifies proteomes from single pre-mRNAs (Ganie & Reddy, 2021). Previous evidence reveals its crucial role in plant stress responses (Zheng et al., 2022). Cold stress significantly impacts AS patterns in cold-responsive genes, contributing to tolerance and adaptation (Zhang et al., 2017; Zheng et al., 2022). Notably, intron retention (IR), the most prevalent AS event in plants, often occurs in stress-responsive genes (Ganie & Reddy, 2021). Unraveling novel AS patterns in cold-tolerant genes will deepen our understanding of abiotic stress responses and pave the way for enhancing plant resilience in extreme environments.

Promoters are upstream DNA sequences of gene-coding regions that play the primary role of regulating the transcriptional initiation of RNA polymerase II (Kor et al., 2023). The core promoter is positioned about 40 bp upstream of the transcription start site (TSS) (Sloutskin et al., 2021). This contains basic elements such as the initiator region (Inr), TSS, cis-acting elements, and TATA box. These elements play an important role in the regulation of gene expression (To et al., 2021; Wang et al., 2021a). The cis-acting elements influence gene expression variation, which is especially important for plant adaptations in their natural environment (Wang et al., 2021a). The polymorphisms of promoters can influence gene expression variations (Savinkova et al., 2023; Wang et al., 2021a). Promoter sequence analysis may reveal details involved in the regulation of gene expression at the transcriptional level, which is essential for increasing our fundamental understanding of gene regulation.

Plant breeding plays a crucial role in developing rice varieties capable of adapting to harsh climatic conditions, thereby ensuring food security and maintaining global rice production potential (Wang et al., 2021b). DNA markers offer significant advantages in breeding programs because they are not affected by the environment and can be used throughout the plant's growth stages (Hasan et al., 2021). Functional markers, specifically, are developed from DNA polymorphisms within genes that directly influence phenotypic variations (Yongbin et al., 2023). This association enables their use in marker-assisted selection (MAS), a powerful tool for identifying desirable traits in breeding lines. Molecular marker analysis techniques, such as PCR and agarose gel electrophoresis, are readily implemented in general laboratories due to their simplicity and cost-effectiveness. In addition, previous research has identified a genomic region from a *japonica* rice cultivar that is potent for enhancing cold tolerance in *indica* varieties (Li & Mao, 2018). This finding paves the way for developing high-yielding *indica* varieties with improved cold resistance, a critical step towards addressing yield losses experienced by *indica* rice in tropical upland areas. Integrating cold tolerance traits into *indica* rice varieties represents a strategic solution for ensuring sustainable food production in these challenging environments.

To gain a better understanding of the genes that play an important role in cold tolerance at the seedling stage in rice, we used RNA sequencing to identify cold-responsive genes in contrasting cold-tolerant and cold-sensitive varieties. This analysis

revealed six genes of potential interest: *OsFH10*, *ONAC045*, *SnRK2*, *OsISA1*, *OsFER1*, and *OsProT*, which exhibited differential expression patterns between the tolerant and sensitive varieties. To further investigate their functional roles in cold tolerance, we explored these genes' expression levels, alternative splicing patterns, DNA methylation status, and promoter sequences. This comprehensive analysis identified notable differences between cold-tolerant and cold-sensitive varieties, suggesting the potential involvement of these genes in the cold-tolerance response. Based on these findings, we successfully developed and validated molecular markers for two of the genes: an SNP marker for *ONAC045* and an InDel marker for *OsProT*. These markers, readily detectable using standard agarose gel electrophoresis, demonstrate potential for application in marker-assisted breeding programs aimed at improving cold tolerance in rice seedlings. Overall, this study provides insights into the genes associated with cold tolerance in rice seedlings. The identification of candidate genes and the development of molecular markers pave the way for the development of cold-tolerant rice varieties in future breeding endeavors.

1.2 Research objectives

1.2.1 To identify genes and possible mechanisms involved in cold tolerance using cold-tolerant and cold-sensitive rice genotypes at the seedling stage by RNA sequencing technology.

1.2.2 To understand the mechanisms involving cold-responsive gene expression in rice at the seedling stage to provide fundamental information on gene regulation under cold stress.

1.2.3 To develop a DNA marker for marker-assisted selection in rice breeding programs aiming to develop cold-tolerant rice varieties.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Rice

Rice stands as one of the world's main food crops, holding an important position in global food security (Gonzalez-Schain et al., 2016). It is the most crucial cereal grain, serving as the primary food source for hundreds of millions residing in tropical and subtropical regions, encompassing Asia, Africa, and Latin America (Yoshida, 1981). Rice is a nutritional powerhouse, containing carbohydrates, protein, vitamins, and minerals, making it an excellent dietary choice (Dwiningsih & Alkahtani, 2022; Oppong et al., 2021). Rice is a staple food for about half of the world's population (Bandumula, 2018; Dwiningsih & Alkahtani, 2022; Zhao et al., 2020). Rice cultivation thrives in Asia, accounting for over 90 % of global production, with *Oryza sativa* L. being the primary species grown there (Bandumula, 2018; Chung et al., 2018). From South Asia to China, Thailand, Japan, and Korea, rice is widespread cultivation (Zhao et al., 2020). Rice occupies a prominent position as Thailand's staple food, cultivated across vast areas encompassing approximately 78.99 million rai nationwide (Chuaykerd et al., 2020). This crop has long served as a cornerstone of the Thai economy, solidifying the country's status as the world's leading rice producer and a prominent exporter to diverse countries of nations across the globe (Chuaykerd et al., 2020; Suwanmontri et al., 2021). Thailand's rice exports reach numerous countries within the Asian continent, including China, Japan, and Southeast Asian nations like Indonesia, the Philippines, and Malaysia. Additionally, Thailand supplies rice to markets beyond Asia, such as Yemen, Israel, and countries bordering the Red Sea (Yusiana et al., 2022).

The cultivated rice plant (*Oryza sativa* L.) is an angiosperm (flowering plant) belonging to the monocotyledon. This classification places it within the *Oryza* genus, further categorized under the *Oryzeae* tribe within the *Poaceae* (*Gramineae*) family (Bulletin, 1965; Kellogg, 2009). The genus *Oryza* exhibits a global distribution across tropical and subtropical regions. It has the adaptability to diverse environmental

conditions, ranging from high mountainous areas to low-land deltas, encompassing an area between 53 °N and 35 °S latitudes. As a cultivated species, *O. sativa* has diverged into three subspecies: *O. sativa* sub sp. *indica*, *O. sativa* sub sp. *japonica*, and *O. sativa* sub sp. *javanica*. According to geography, evolution occurs as a result of long-term adaptation to various climatic conditions. Comprising 12 chromosomes, the *Oryza* genome encompasses 389 Mb and encodes approximately 32,000 genes (Sweeney & Mccouch, 2007; Zhang et al., 2020).

The rice plant is an annual grass that can grow to a height vary considerably, ranging from a mere 0.3 meters to 7 meters. The root system is fibrous, and the main stem (culm) is segmented into nodes and internodes. Each node bears a leaf and a bud. The leaf itself is comprised of a sheath and a blade. The flowers, containing stamens and pistils, are arranged in a branching structure called a panicle, which emerges from the uppermost internode of the culm (Bulletin, 1965). The two major subspecies of Asian cultivated rice (*Oryza sativa* L.) are *indica* and *japonica*. These two subspecies have been geographically separated due to their distinct environmental adaptations. *indica* varieties are predominantly cultivated in subtropical and tropical regions at low latitudes, while *japonica* varieties are primarily cultivated in temperate regions at high latitudes. This geographical separation has driven genetic divergence between the two subspecies, resulting in differential gene expression patterns that are crucial for their adaptation to respective environments (Wang et al., 2021).

Abiotic-tolerant germplasm is a valuable genetic resource for the development of new climate-resilient varieties in future rice breeding programs (Kanngan et al., 2023). The identified germplasm that has high cold tolerance can be beneficial to developing novel varieties with enhanced cold tolerance (Dar et al., 2021). The search for new sources of tolerant genes becomes imperative for the breeding program. Single-nucleotide polymorphisms (SNPs) are one of the materials with high potential for the molecular breeding of rice (Gonzaga et al., 2015). Marker-assisted selection is a practical technique to quicken the breeding process (Hassan et al., 2023). Molecular markers have been widely used in breeding programs, providing advantages with no environmental impact (Hasan et al., 2021; Yongbin et al., 2023).

2.2 Cold stress effects

Rice is susceptible to various environmental stresses, including drought, salinity, and cold. Among these, cold stress significantly threatens rice cultivation, especially at low temperatures (Amien et al., 1999; Ma et al., 2015). Cold stress adversely impacts rice growth and seed setting rates, reducing overall productivity (Ye et al., 2009). *Indica* rice, the predominant variety cultivated in Thailand, is generally more sensitive to cold stress compared to *japonica* rice, likely due to its tropical variety (Dasgupta et al., 2020; Yang et al., 2015). Rice plays a crucial role in Thailand's agricultural economy (Suwanmontri et al., 2021; Wongwaiwech et al., 2019). However, rice cultivation in Thailand faces increasing challenges due to the rising frequency of cold weather events. Thailand experiences a cold spell in the country beginning in October, particularly in December and January, when temperatures are at their lowest (World Bank Group, 2021). Cold stress exerts a multifaceted impact on rice growth and production, posing a significant threat to food security. Cold stress poses a significant hurdle to rice cultivation, adversely influencing various growth stages, from germination to grain formation (Guo et al., 2019).

Cold stress exposure in plants triggers a cascade of physicochemical disturbances that collectively hamper plant growth and development. These disturbances encompass the disruption of micro-organelles, disintegration of the plasma membrane, denaturation of essential macromolecules, and dysfunction of the electron transfer chain (ETC). Additionally, cold stress accelerates the degradation of crucial macromolecules such as polysaccharides, lipids, photosynthetic pigments, enzymes, and nucleic acids. Furthermore, cold stress intensifies reactive oxygen species (ROS) production, malondialdehyde (MDA) accumulation, and electrolyte leakage (EL), while concomitantly suppressing photosynthesis, membrane-bound enzyme activity, nitrogen uptake capacity, and general metabolic processes (Shahandashti & Amiri, 2018; Wang et al., 2023; Yadav, 2010; Ye et al., 2009; Zhang et al., 2014). These cumulative disruptions manifest as visible injury symptoms, including chlorosis (leaf yellowing), necrosis (tissue death), poor germination, stunted seedlings, withering, reduced tillering, pollen sterility, and irregular panicles, collectively contributing to significant yield reductions (Yadav, 2010). The cumulative effects of these injury

symptoms hinder plant growth and development, leading to overall growth inhibition (Yadav, 2010; Ye et al., 2009; Zhang et al., 2014). In response to cold stress, plants activate transcription factors and cold-responsive genes, leading to elevated antioxidant production. Antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) scavenge ROS to protect plants from oxidative damage. Additionally, plants remodel the plasma membrane and accelerate osmolyte synthesis to mitigate cold stress-induced damage (Ma et al., 2015; Wang et al., 2023; Yadav, 2010).

Indica rice, a tropical subspecies of *Oryza sativa*, exhibits greater susceptibility to cold stress compared to *japonica* rice (Dasgupta et al., 2020; Ham et al., 2021; Zhang et al., 2012). This differential sensitivity stems from distinct habitat adaptations between *indica* and *japonica* rice, resulting in fundamental and differential gene expression patterns in response to various environmental stresses, including cold, drought, and salinity (Wang et al., 2021).

Cold tolerance in rice is a complex trait governed by an abundance of genes, and extensive research has led to the identification of numerous cold-tolerance-related quantitative trait loci (QTLs). Over the past two decades, progress has been made in identifying these QTLs, including *qCTP11* associated with cold tolerance at the germination stage, qCtss11 and qCTS4a associated with cold tolerance at the seedling stage, and *Ctb1*, *qCTB2a*, *qPSST-3*, and *qLTB3* associated with cold tolerance at the reproductive stage (Zhang et al., 2014; Zhou et al., 2010).

2.3 Rice seedling

The seedling stage represents one of the most sensitive stages to cold stress. Exposure to cold stress during the seedling stage is a significant threat to rice productivity, having lower rice biomass and being injured, particularly in tropical and subtropical regions (Dasgupta et al., 2020; Wang et al., 2023; Zhang et al., 2012). Cold stress during the seedling stage can manifest in various detrimental effects, including stunted growth, non-uniform development, delayed maturation, reduced tillering, and weak seedlings, ultimately leading to substantial yield losses (Dong et al., 2019; Ye et al., 2009). Leaf rolling and discoloration, two prominent chilling injury symptoms, are

commonly used as visual indicators for evaluating cold tolerance at the seedling stage (Dong et al., 2019). Given the critical role of the seedling stage in determining overall cold tolerance, seedling survival rate (%) is frequently employed as a key metric for assessing cold tolerance in rice cultivars (Zhang et al., 2017). Consequently, breeding for enhanced cold tolerance at the seedling stage is of paramount importance for improving rice production in tropical and subtropical regions. Enriching the cold tolerance of *indica* rice cultivars, which are predominantly cultivated in these regions, holds the potential for mitigating yield losses and ensuring food security (Dasgupta et al., 2020: Zhang et al., 2012).

2.4 Transcriptomes

Transcriptomes represent gene expression profiles at the transcriptional level, serving as valuable indicators of gene activity and the intricate molecular mechanisms underlying specific biological processes, including gene structure, regulation of gene expression, gene product function, and genome dynamics (Adams, 2008; Dong & Chen, 2013). RNA sequencing technology has emerged as a powerful approach for transcriptome profiling by utilizing deep-sequencing techniques (Wang et al., 2009). It unravels gene networks associated with multiple genes, a consequential outcome of transcriptomic changes in plant response mechanisms (Pan et al., 2020). The application of RNA sequencing to the rice genome has generated a wealth of transcriptome data under various abiotic stresses (Kumar & Dash, 2019). Additionally, multiple mechanisms, such as promoters, alternative splicing, and DNA methylation, contribute to the regulation of gene expression (Anaya et al., 2013).

Shen et al. (2014) performed RNA sequencing to identify novel genes and uncover potential mechanisms underlying chilling tolerance responses in rice seedlings. Their findings revealed a total of 2242 commonly regulated differentially expressed genes (DEGs) in response to cold stress across all genotypes, while 318 DEGs emerged as common DEGs associated with cold tolerance in the three cold-tolerant genotypes. Functionally, these 318 cold tolerance-related DEGs implicated the involvement of components related to the reduction of functional photosynthesis proteins, alterations in epigenetic processes, and compensation of ATPase activity.

Yang et al. (2015) undertook a comparative transcriptome analysis of cold stress responses in the shoots and roots of two rice seedlings, TCN1 (cold-sensitive) and TNG67 (cold-tolerant). Their gene ontology and pathway analyses unveiled a significant impact of cold stress on the expression of genes associated with protein metabolism, modification, translation, stress responses, and cell death.

Do Amaral et al. (2016) utilized RNA sequencing technology to compare the transcriptional profiles of rice plants subjected to cold stress, identifying 7,905 DEGs. Functional annotation revealed that cold stress predominantly induced the most significant alterations in overall metabolism. Enrichment analysis of overrepresented gene ontology (GO) terms indicated that the majority of these genes are localized to plastids, ribosomes, and chloroplasts.

2.5 DNA methylation

Epigenetics encompasses the study of changes in gene expression at the transcriptional level without altering the underlying DNA sequence (Barozai & Aziz, 2018; Kumar, 2018; Moore et al., 2013; Yaish, 2013). This intriguing mechanism enables cells with identical genetic material to exhibit distinct gene expression patterns (Moore et al., 2013). Epigenetic modifications play a crucial role in organismal adaptation to environmental changes (Çelik et al., 2019; Kumar, 2018). The major epigenetic modifications include DNA methylation, histone modifications that regulate chromatin conformation, and non-coding RNAs (Howe, 2018).

DNA methylation is the process of adding a methyl group to the C5 position of the cytosine pyrimidine ring in DNA, resulting in the formation of 5-methylcytosine (Derks et al., 2004; He et al., 2011; Moore et al., 2013). This fundamental process plays a crucial role in numerous cellular processes, including the regulation of gene expression through transcriptional repression (Moore et al., 2013; Phillips, 2008). CpG islands are DNA sequences with a higher CpG density and fewer nucleosomes compared to other genomic regions. The majority of cytosine methylation occurs within CpG islands (Moore et al., 2013). Promoters of genes often reside within CpG islands, where DNA methylation can lead to transcriptional silencing and the suppression of gene expression (Lim et al., 2019; Moore et al., 2013). Methylation of CpG islands

within promoter regions interferes with the binding of transcription factors to DNA, thereby inhibiting gene transcription (Figure 2.1) (Howe, 2018; Moore et al., 2013).

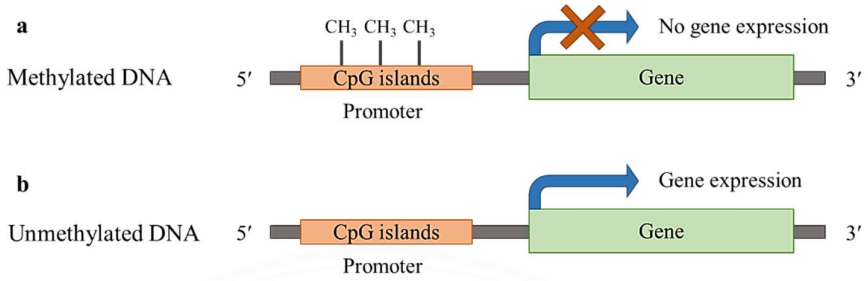


Figure 2.1 The model delineates the differential regulatory effects of methylated and unmethylated CpG islands on gene expression. **a**) Methylated CpG islands within promoter regions hinder gene expression by impeding the binding of transcription factors to DNA, effectively silencing gene transcription. **b**) Unmethylated CpG islands in promoter regions permit the binding of transcription factors, facilitating gene expression.

DNA methylation stands as a critical epigenetic mechanism underlying plant stress responses by modulating gene expression patterns (Lim et al., 2019; Pan et al., 2011). This dynamic process serves as a protective mechanism, modifying gene expression to defend plants from environmental stress-induced damage (Yaish, 2013). Rice seedlings exposed to cold stress (2–3 ºC) exhibited genome-wide DNA methylation alterations, suggesting that these changes are influenced by growth stage, tissue type, and rice variety (Pan et al., 2011).

Methylation-specific PCR (MSP) offers a simple, rapid, and cost-effective approach for analyzing the DNA methylation status of CpG islands (Derks et al., 2004; Herman et al., 1996). This technique involves two steps: sodium bisulfite-mediated DNA modification and PCR amplification using specific primer sets to detect differences between methylated and unmethylated alleles (Fraga & Esteller, 2002; Herman et al., 1996). Understanding the DNA methylation patterns of CpG islands associated with cold-tolerant genes is crucial for understanding gene regulation mechanisms that facilitate cold-stress adaptation. This knowledge will be useful for

future applications aimed at enhancing cold tolerance in rice seedlings through genetic manipulation.

2.6 Alternative splicing

Alternative splicing (AS) is a post-transcriptional gene regulatory mechanism that diversifies the proteome from a single gene by selectively including or excluding different exons during pre-mRNA processing (Leviatan et al., 2013; Ganie & Reddy, 2021; Rosenkranz et al., 2022). This dynamic process generates transcripts that encode distinct protein isoforms with unique functions, properties, localization, or stability, significantly enhancing the complexity of eukaryotic transcriptomes and proteomes (Gao et al., 2023; Rosenkranz et al., 2022; Shang et al., 2017). AS plays a crucial role in regulating gene expression and transcript abundance at the post-transcriptional level (Rosenkranz et al., 2022; Shang et al., 2017). Splicing is a post-transcriptional RNA modification process that involves the precise removal of introns and the ligation of exons to generate mature RNA transcripts (Tian, 2013). This process is driven by the interplay of trans-acting factors that recognize specific cis-regulatory elements within the pre-mRNA (Rosenkranz et al., 2022). Common AS events include exon skipping, intron retention, alternative 5' splice site selection, and alternative 3' splice site selection (Figure 2.2) (Leviatan et al., 2013; Li et al., 2020; Rosenkranz et al., 2022; Syed et al., 2012).

AS is a critical transcriptional regulatory mechanism that plays a pivotal role in plant survival and environmental adaptation (Rosenkranz et al., 2022; Shang et al., 2017). Mounting evidence suggests that AS serves as a crucial regulator of gene expression in plant responses to diverse stress conditions (Leviatan et al., 2013; Wei et al., 2020; Zheng et al., 2022). AS functions as an essential regulatory mechanism in rice, undergoing significant alterations in response to various environmental stresses, enabling rapid reprogramming of gene expression to enhance plant survival under stress conditions (Ganie and Reddy, 2021). Studies on the effects of cold on gene expression levels indicate that cold stress modulates the expression of pre-mRNA splicing in cold-responsive genes (Leviatan et al., 2013). Notably, AS has been implicated in playing a crucial role in cold tolerance and plant adaptation during cold exposure (Chinnusamy

et al., 2007; Zheng et al., 2022). Zhang et al. (2017) demonstrated that long-term cold stress induces AS events in rice seedlings and reproductive stages, with the majority occurring in up-regulated genes, suggesting that cold-induced AS contributes to cold stress tolerance. AS events have an expression pattern specific to genotypes and genes (Ganie and Reddy, 2021).

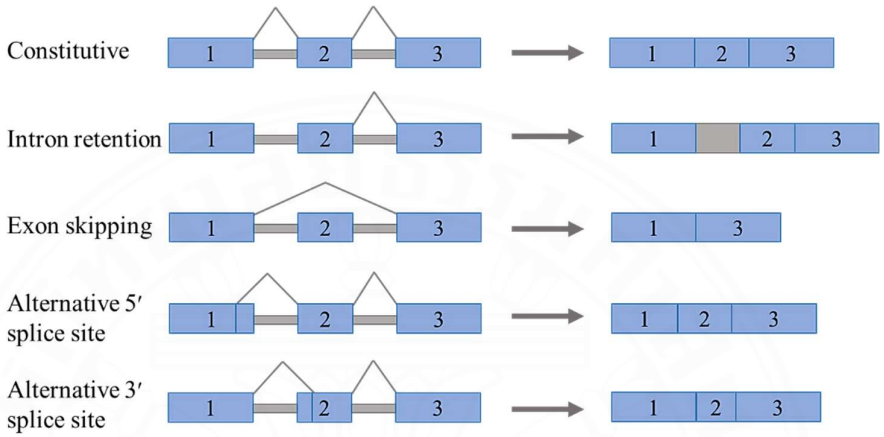


Figure 2.2 AS events include exon skipping, intron retention, alternative 5' splice site selection, and alternative 3' splice site selection. Exons are represented by blue boxes, introns by gray lines, and splicing patterns by diagonal lines.

Among all AS events, intron retention (IR) is the most prevalent in plants, often occurring in genes associated with stress responses (Ganie & Reddy, 2021; Shankar et al., 2016). Previously, IR was considered a splicing error that resulted in the retention of non-coding introns, leading to the production of truncated, non-functional proteins. Consequently, IR received limited attention in the field of plant biology. However, recent studies have demonstrated the critical role of IR in regulating plant physiology. IR plays a role in regulating gene expression programs and potentially influences rice's ability to tolerate stress (Ganie & Reddy, 2021). Elucidating the mechanisms underlying IR and identifying novel AS events will deepen our understanding of how splicing patterns impact plant abiotic stress tolerance, paving the way for innovative strategies to enhance plant resilience to extreme environments.

2.7 Promoters

Promoters are upstream DNA sequences located in gene-coding regions. They play a pivotal role in regulating the initiation of RNA polymerase II-mediated transcription (Biłas et al., 2016; Haberle & Stark, 2018; Garcia & Finer, 2014). Compared to prokaryotic promoters, eukaryotic promoters are larger and more complex in their structure (Thrall & Goodrich, 2013; Zhou et al., 2017). The core promoter, proximal promoter, and distal promoter are the three main components of typical promoter structures (Aysha et al., 2018; Zhang, 2007). Core promoters act as specific DNA sequence-binding platforms flanking the transcription start site (TSS). They facilitate the assembly of the transcriptional machinery necessary for gene expression (Haberle & Stark, 2018). The core promoter typically resides within 40 base pairs upstream and downstream of the TSS (Thrall & Goodrich, 2013). Fundamental components of the core promoter include the TATA box, the initiator region (Inr), and cis-regulatory elements (Figure 2.3). These elements play crucial roles in regulating gene expression (To et al., 2021; Wang et al., 2020). The TATA box serves as a binding site for the TATA-binding protein (TBP), a subunit of the transcription initiation factor complex (Garcia & Finer, 2014; Haberle & Stark, 2018). Approximately 8 base pairs in length, the TATA box motif is located roughly 24-31 base pairs upstream of the TSS and exhibits a conserved sequence across species, from yeast to humans (Haberle & Stark, 2018; Thrall & Goodrich, 2013). Promoters of numerous highly expressed genes often retain a strong TATA box (Solovyev et al., 2010). The TSS, which binds TBP-associated factors 1 and 2 (*TAF1* and *TAF2*), is located within the Inr (Thrall a& Goodrich, 2013). While not universal, the Inr is more common than the TATA box and its consensus sequence varies between species. In promoters lacking a TATA box, the Inr is often accompanied by another motif, such as the downstream promoter element (DPE) (Haberle & Stark, 2018). The TSS is typically located within the Inr, which resides approximately 25-30 base pairs downstream of the TATA box (Biłas et al., 2016; To et al., 2021). The TSS is situated within a short sequence called the core promoter, encompassing approximately 50 base pairs upstream and 50 base pairs downstream of the TSS (Haberle & Stark, 2018). Multiple cis-regulatory elements, non-coding DNA sequences found upstream of the TSS within promoters, regulate the

transcriptional effect on plant phenotypes during development (Biłas et al., 2016; To et al., 2021). Notably, cis-elements influence gene expression variation, which is critical for plant adaptations to their natural environment (Wang et al., 2020). Additional motifs found downstream of the TSS include the downstream core element (DCE), the motif ten element (MTE), and the downstream promoter element (DPE) (Thrall & Goodrich, 2013). Regulatory sequences in promoters, such as enhancers and silencers located in the proximal or distal regions, contribute to the control of gene expression by repressing or activating transcription (Solovyev et al., 2010; Garcia & Finer, 2014). Analyzing promoter sequences can provide valuable insights into the mechanisms governing gene expression at the transcriptional level. This understanding is crucial for advancing our fundamental knowledge of gene regulation.

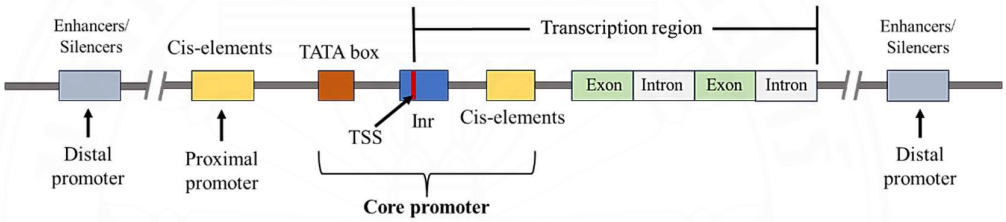


Figure 2.3 The model gene promoter structure, the core promoter region comprises the TATA box, the initiator region (Inr), the transcription start site (TSS), and cis-regulatory elements.

2.8 Molecular markers

Plant breeding plays a vital role in enhancing the ability of rice varieties to tolerate extreme climatic conditions, thereby supporting food sustainability and maintaining long-term productivity potential (Dar et al., 2021; Juma et al., 2021). Molecular markers are defined as any detectable characteristic that reveals variations in the DNA nucleotide sequence, indicating genetic polymorphisms within or between species or organisms. The observed variations in DNA markers arise from various types of mutations, including point mutations (substitutions), rearrangements (insertions or deletions), and errors during DNA replication (Kordrostami & Rahimi, 2015; Amiteye,

2021). These polymorphisms are categorized into three main classes based on their detection methods: hybridization-based (RFLP), PCR-based (RAPD, SSR, SNP), and hybridization combined with PCR (AFLP) (Vishwakarma et al., 2022). Variations in the DNA nucleotide sequence can be associated with specific genes responsible for certain traits. The marker and the associated gene are also inherited together (Amiteye, 2021). Molecular markers have emerged as valuable tools in genetic analysis and plant breeding research, accelerating breeding progress and enhancing the efficiency of developing new crop varieties with desirable traits (Amiteye, 2021; Hasan et al., 2021; Hassan et al., 2023; Kordrostami & Rahimi, 2015). Implementing marker-assisted selection (MAS) in breeding programs significantly improves the selection process by directly targeting specific genes associated with desired phenotypes (Hasan et al., 2021). Plant breeders utilize molecular markers for various applications, including fingerprinting germplasm, assessing genetic diversity within and between cultivars, and mapping quantitative trait loci (QTLs) (Kordrostami & Rahimi, 2015). MAS has demonstrated its effectiveness in enhancing diverse traits across various crops, including those related to biotic and abiotic stress resistance and quality attributes (Vishwakarma et al., 2022). DNA markers possess several advantages over traditional breeding methods, including being unaffected by environmental factors and observable at all stages of plant development and in all tissues and organs (Amiteye, 2021; Hasan et al., 2021; Kordrostami & Rahimi, 2015). A desirable DNA marker should exhibit several key characteristics, including co-dominance (allowing discrimination between homozygotes and heterozygotes), high polymorphism, uniform distribution across the genome, high reproducibility, and the ability to share data across laboratories. PCR-based markers have gained widespread popularity in the variation analysis of plant genomics due to their ability to specifically target DNA regions using primer pairs (Amiteye, 2021; Hasan et al., 2021). Examples of widely used PCR-based markers include EST-SSRs, AFLPs, RAPDs, RAMPs, SRAPs, ISSRs, and SCARs (Hasan et al., 2021). Utilizing PCR-based techniques and agarose gel electrophoresis for DNA marker analysis offers a simple and accessible method for detecting genetic variations in conventional laboratory settings. Differences in band sizes on the gels represent marker alleles (Amiteye, 2021).

Single nucleotide polymorphisms (SNPs) and insertions/deletions (InDels) represent the most common types of DNA sequence variations, found abundantly within the genomes of all species. These markers have gained widespread application in MAS due to their abundance (Feng et al., 2021; Li et al., 2023). The advancement of next-generation sequencing technologies has significantly accelerated the development and application of SNP and InDel markers in plant breeding research (Hu et al., 2020; Li et al., 2023; Long et al., 2022). SNPs are genetic polymorphisms arising from single nucleotide differences in DNA sequences, typically caused by point mutations between individuals within or across species (Amiteye, 2021; Vishwakarma et al., 2022). They are extensively utilized in plant research and have garnered increased interest following the publication of numerous plant genome sequences (Vishwakarma et al., 2022). With a frequency of approximately one SNP per 100-300 base pairs of DNA, SNPs represent the most frequent type of DNA sequence polymorphism across organisms (Amiteye, 2021). Their high abundance and informativeness make them valuable resources for plant molecular breeding (Gonzaga et al., 2015). Numerous techniques are currently available for SNP genotyping (Amiteye, 2021), with sequencing or fluorescence-based techniques (Amiteye 2021; Lee et al., 2022; Yang et al., 2019). Furthermore, InDel markers, exhibiting size variations exceeding 10 base pairs, have emerged as valuable materials for rice molecular breeding due to their ease of detection and analysis (Long et al., 2022). InDel markers have gained increasing attention in plant genetic studies, demonstrating their effectiveness in marker-assisted breeding strategies (Li et al., 2023; Long et al., 2022). Promoter sequences, which assemble several transcription factor binding sites, are critical in initiating gene expression and exhibit high diversity (Hasan et al., 2021). Polymorphisms within these regions can influence variations in gene expression levels (Wang et al., 2020). MAS represents a powerful strategy for enhancing cold tolerance phenotypes. Several studies have reported the successful application of MAS to improve cold tolerance in plants (Hasan et al., 2021).

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Plant materials and Sample collection

Five rice varieties (*Oryza sativa* L.) were used to observe responses to cold stress (Yongbin et al., 2023). They were two extremely cold-tolerant varieties (*Nipponbare*, NB, and B30, IR14632-2-3), one moderately cold-tolerant variety (DPY), and two cold-sensitive varieties (RD31 and SPR90). NB is a *japonica* variety. B30 was obtained from the International Rice Research Institute (IRRI). RD31 and SPR90 are Thai *indica* varieties. First, the responses of seedlings to cold stress were evaluated in all varieties to find the time point showing distinct phenotypic differences between cold-tolerant and cold-sensitive varieties. Thirty rice seeds of each variety were soaked in water for 24 hours, then drained and allowed to germinate in the dark at 32–35 ºC for 24 hours. Good-germinated seeds were planted in small plastic bags filled with fine field soil (15 seeds per bag). For each variety, 2 bags were prepared for cold stress (CS) (6-8 °C) and control (CT) (30±3 °C) conditions. The seedlings were grown in a greenhouse at National Science and Technology Development Agency (NSTDA), Thailand under natural condition until they obtained the three-leaf stage (10-11 days). When seedlings reached the three-leaf stage, they were used for a cold-stress treatment. For each variety, one bag of seedlings was placed in a cold (6-8 ºC) chamber, while the other bag was placed in a control (30±3 °C) chamber. The seedlings were grown at 12 hours light and 12 hours dark photoperiods.

The responses of seedlings to cold stress were observed at 3, 12, 24, 48, and 72 hours after the treatment. At 72 hours after the treatments, all treatments were transferred to natural conditions for a week. After that, the survival rate was calculated by seedling survival rate (%) = (surviving seedlings × 100) / total seedlings.

For sample collection, seedlings of the five varieties were grown and treated in the same manner as described for cold treatment. Seedlings were treated with cold and control treatments for 24 hours, when cold-tolerant and cold-sensitive varieties showed distinct phenotypes. Shoots of seedlings in both treatments were collected and

kept in a 1.5 mL microcentrifuge tube. The seedling samples were frozen in liquid nitrogen and stored in ultra-freezer at -80 °C until they were used for RNA extraction.

3.2 RNA extraction

For RNA extraction, the bulk of three seedlings from each variety and treatment were used. The tissues were homogenized with liquid nitrogen. The total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentration and purity of RNA were then determined using a nanodrop spectrophotometer. RNA degradation and contamination were evaluated on 1 % agarose gels. Finally, high-quality RNA was stored at -80 °C for RNA sequencing and validation by RT-PCR.

3.3 RNA sequencing and data analysis

For RNA sequencing, three rice varieties were chosen as representatives in this study, including one cold-tolerant variety (B30) and two cold-sensitive varieties (RD31 and SPR90). The total RNA of three rice varieties was submitted to Novogene Bioinformatics Technology Co., Ltd. for RNA sequencing, following the manufacturer's recommendations. RNA sequencing consists of three steps, including library preparation, sequencing, and data analysis. Each sample's libraries were prepared using the RNA Library Prep Kit for Illumina. After that, all sample libraries were sequenced on an Illumina platform. Raw reads from sequencing were filtered to clean reads, and then paired-end clean reads of each sample were mapped to the reference genome. The differentially expressed genes between the conditions of each variety were analyzed using the DEGSeq R package. A corrected P-value of 0.05 and log2 (fold Change) of 1 were set as the thresholds for significantly differential expression.

3.4 Validation of differential gene expression

DEGs identified from RNA sequencing data were validated using semi-quantitative RT-PCR. For this study, the twelve DEGs were selected based specifically on up-or down-regulated in the B30 variety but not in the RD31 or SPR90 varieties. Furthermore, these DEGs have a high log2 fold change and a more read court. Specific primers were designed by the Primer3 program, and the gene sequences were obtained from the Gramene database. The primer sequences are detailed in Table 4.3. Gene validation was performed with RNA samples of five varieties (NB, B30, DPY, RD31, and SPR90). Complementary DNA was synthesized using total RNA. A total amount of 1 µg RNA per sample was carried out using reverse transcription with iScript cDNA Synthesis Kit (Bio-RAD, USA) in a 10 µl PCR reaction according to the manufacturer's instructions. Subsequently, cDNA was diluted 5-fold and used as a PCR template. The quantity of template cDNA was adjusted in this analysis by quantifying the expression of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene as a reference gene.

Semi-RT-PCR was performed on diluted cDNA (2 µl) using GoTaq™ G2 green master mix (Promega, US) and gene-specific primers. The PCR reactions were performed as follows: denaturation at 94°C for 5 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min, with a final elongation step of 72 °C for 7 min. The PCR processes were carried out in 24-32 cycles to ensure that the amount of the amplified products could be distinguished between different levels of gene expressions based on concentrations of DNA band. All PCR products were electrophoresed on 1.5 % agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. Each set of experiments was repeated three times. The PCR products of the semi-RT-PCR were confirmed by sequencing.

DEGs that show different expression patterns between cold-tolerant and sensitive varieties were selected to confirm their expressions by quantitative real-time PCR (qRT-PCR). *OsFH10, ONAC045, SnRK2, OsISA1, OsFER1,* and *OsProT* were selected to confirm by qRT-PCR. qRT-PCR was performed with RNA samples of three varieties (B30, RD31, and SPR90) with qPCRBIO SyGreen Mix Lo-ROX. Each reaction was performed as follows: denaturation at 95 °C for 3 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The relative expression

levels were calculated using the ΔΔCq method with *GAPDH* as the reference gene. Then, interesting DEGs were selected for analysis of gene regulation, including DNA methylation, alternative splicing, promoter sequence and marker development.

3.5 Methylation status analysis by methylation specific PCR (MSP)

The MSP technique detects the average DNA methylation status of a gene using primer sets, including bisulfite (B), methylated (M), and unmethylated (U) specific primers. The promoters of genes were searched in the Gramene database for CpG island analysis with GC content greater than 50 %. *OsFH10* was selected to study their methylation status. Because *OsFH10* has a clear distinct expression in cold-tolerant and sensitive varieties. Meth-Primer 2.0 software, a free online tool was used to detect CpG islands in the prompter of the selected genes. After that, the Meth-Primer Program was used to design MSP primer sets for each locus based on the sequence of CpG islands. The primer sequences are detailed in Table 4.3. The B primer detects a complete bisulfite treatment. The M primer detects methylated DNA. The U primer detects unmethylated DNA.

Seedling samples from five varieties (NB, B30, DPY, RD31, and SPR90) under cold and control treatments were used for MSP experiments. Total genomic DNA was extracted from each seedling sample and stored at -20 °C. The genomic DNA from each sample was modified with sodium bisulfite using an EZ DNA methylation kit according to the manufacturer's instructions. After modification, cytosine (C) in genomic DNA is converted to uracil (U) and finally transformed into thymine (T), but this does not occur with 5-methylcytosine (5-mc). The modified DNA was utilized as a template for the MSP using gene-specific primers for genes. PCR reactions were performed by MSP primer sets of each gene with GoTaq™ G2 Green Master Mix under the following conditions: initiated step of 5 min at 94 °C (one cycle only), followed by 40 to 45 repeated cycles of 94 °C for 45 s, 55 to 63 °C for 45 s, 72 °C for 1 min s, and a final extension at 72 °C for 10 min. The analysis of the PCR products on a 2 % agarose gel electrophoresis was used to investigate the average methylation status of the genes. The results were presented as presence or absence, and the PCR reactions were repeated three times.

3.6 Alternative splicing

To gain insight into potential alternative splicing events, we employed semi-quantitative PCR techniques to analyze the expression of the six selected genes. These included the *OsFH10, SnRK2, OsISA1, OsFER1, ONAC045,* and *OsProT* genes (Table 4.4). These genes present specific expression patterns in cold-tolerant varieties. The bam file from the RNA sequencing B30 result was visualized by the integrative genomics viewer (IGV) program. Junction tracks show arcs connecting alignment reads that are InDels in a gene, and coverage tracks show read coverage in the gene region. Primers specifically designed to detect AS events were developed utilizing structural data of the respective genes from Gramene (http://www.gramene.org/) using the Primer3 program. The specific primers were designed to cover 2–3 introns, resulting in different DNA sizes showing alternative splicing events. For the *OsFH10* gene, the first set of AS primers, FH10-IsF1, targeting exon1-exon3 and intron1 - intron2, amplify a fragment ranging from 813 to 1238 bp in size. The second set, FH10-IsF2, targets exon3 - exon6 and intron3 - intron5, amplifying fragments ranging from 707 to 1,376 bp. For *the SnRK2* gene, SnRK2-IsF targets exons2 – exons7 and intron2 – intron6 amplifying fragments ranging from 1155 to 1672 bp. Two primer sets were designed for the *OsISA1* gene. The first set, ISA1-IsF1, targets exon1 – exon4 and intron1 – intron3, amplifying a fragment between 476 and 1105 bp, while the second set, ISA1-IsF2, targets exon11 – exon14 and intron11 – intron13, amplifying fragments ranging from 348 to 1713 bp. AS primers for the *OsFER1* gene, FER1-IsF, targeting exons1 - exons4 and intron1 - intron3 amplify a fragment between 243 and 825 bp. AS primers designed for the *ONAC045* gene, NAC045-IsF, targeting exon1 - exon3 and intron1 - intron2 amplify a fragment ranging from 644 to 941 bp. Two primer sets were designed for the *OsProT* gene. The first set, ProT-IsF1, targets exon2 - exon5 and intron2 - intron4, amplifying a fragment between 475 and 1473 bp, while the second set, ProT-IsF2, targets exon5 - exon8 and intron5 - intron7, amplifying fragments ranging from 713 to 10930 bp. When AS events occur, this primer can generate a PCR product that detects and distinguishes between the normal isoform and the AS isoform on agarose gel electrophoresis due to different sizes. The primer sequences are detailed in Table 4.3.

Three cold-tolerant cultivars (NB, B30, and DPY) and two cold-sensitive cultivars (RD31 and SPR90) were included in this study. Complementary DNA (cDNA) was synthesized using total RNA. A total amount of 1 µg RNA per sample was carried out using reverse transcription with iScript cDNA Synthesis Kit (Bio-RAD, USA) in a 10 µl PCR reaction according to the manufacturer's instructions. Subsequently, cDNA was diluted 5-fold and used as a PCR template. The quantity of template cDNA was adjusted in this analysis by quantifying the expression of the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene as a reference gene. Diluted cDNA from five varieties in both conditions served as the template for amplification using gene-specific AS primers and GoTaq™ G2 green master mix. The PCR reactions were conducted as follows: 94 °C denaturation for 5 minutes, followed by 30-35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min, with a final elongation step at 72 °C for 7 min. Subsequently, all PCR products were electrophoresed on a 2 % agarose gel, stained with ethidium bromide, and visualized under a UV lamp. Each experimental set was repeated three times to ensure reproducibility. Transcript isoforms were identified based on PCR size differences.

3.7 Promoter sequence analysis

To investigate potential regulatory differences between cold-tolerant and cold-sensitive varieties, the promoters of candidate genes exhibiting upregulation were sequenced using DNA from both. Expression analysis confirmed upregulation in cold-tolerant varieties, while cold-sensitive varieties exhibited no, differential, or downregulated expression. Approximately 700-1000 bp upstream sequences, encompassing the putative transcription start site (TSS) and potential promoter binding sites, were retrieved from the Gramene database (http://www.gramene.org/) and used for primer design using Primer3 software. The primer sequences are detailed in Table 4.3. These primers yielded PCR products of approximately 700-800 bp.

Promoter sequence analysis was performed on three rice varieties: B30, RD31, and SPR90. Seeds from each variety were grown to the three-leaf stage, and genomic DNA was extracted from young leaf tissue using the cetyltrimethylammonium bromide (CTAB) method. DNA quality was assessed by Nanodrop and stored at -20 °C

until PCR analysis. PCR amplification was conducted using GoTaq™ G2 green master mix and gene-specific primers under the following conditions: 94 °C denaturation for 5 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 70 °C for 2 min, with a final elongation step at 72 °C for 7 min. All PCR products were purified with GenepHlow™ Gel/PCR Kits and subsequently sent for sequencing.

Promoter sequences for each gene were assembled into contigs using BioEdit and aligned with corresponding sequences from reference cultivars NB and 9311 in ClusterW. This alignment identified divergent sequences between the cold-tolerant (NB and B30) and cold-sensitive (9311, RD31, and SPR90) groups. Subsequently, the TSSP program (http://www.softberry.com/) with default settings was employed to predict the transcriptional start site (TSS), while PlantCare identified potential functional elements within the promoter sequences.

3.8 Marker Development

OsProT and *ONAC045* were selected to develop SNP and InDel markers, respectively, due to the differences in gene expression between the cold-tolerant and cold-sensitive varieties, and the available SNP and InDel markers suitable for markers development.

For *ONAC045*, G/A SNP was located near the TATA-box, an important location for gene expression. The G/A mutations were distinguished using the tetra-primer amplification refractory mutation system (T-ARM)-PCR, which results in different alleles in PCR reactions. The sequences surrounding the G/A mutation were used to design allele-specific primers through the PRIMER1 program (http://primer1.soton.ac.uk/primer1.html). The primer sequences are detailed in Table 4.3. The 200 - 300 bp PCR products were developed to ensure easily observe polymorphisms between genotypes using agarose gel electrophoresis. Amplification with an inner F primer and an outer R primer indicates the G allele and amplification with an outer F primer and an inner R primer indicates the A allele. This marker was used to genotype ten cold-tolerant and ten cold-sensitive varieties to assess polymorphism and optimize the PCR conditions. Then, the marker was used to genotype the germplasm for validation. For the PCR analysis, GoTaq^{TM} G2 Green Master Mix

(Promega, U.S.A.) were used in 10 µL PCR reaction, including 2 µL (10-100 ng) of DNA template, 5 µL of 2x Master Mix, 1 µL of each inner primer (10 nM), 0.5 µL of each outer primer (10 nM). The PCR reactions were performed as follows: denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min, with a final elongation step of 72 °C for 7 min. The PCR products were analyzed by size on 2 % agarose gel electrophoresis for 60 min at 90 V in TBE buffer with a 100 bp DNA ladder.

Because *japonica* and *indica* have cold-tolerant and cold-sensitive varieties, respectively (Cen et al., 2018). The *OsProT* sequences from Nipponbare (*japonica*) and 9311 (*indica*) were aligned using the Clustal W program, resulting in an InDel of 82 bp difference between the two subspecies. This difference could be associated with the cold-tolerant process. Thus, the 82 bp was used for the InDel marker development using the flanking sequences on both sides of the InDel using the Primer3 online primer design tool. The primer sequences are detailed in Table 4.3. The pair of primers covered the InDel region, resulting in approximately 200 - 300 bp of PCR product to simple identify polymorphisms using agarose gel electrophoresis. The obtained marker was used to genotype ten cold-tolerant and ten cold-sensitive varieties to assess polymorphism and to optimize the PCR conditions. After that, the marker was used to genotype the germplasm for validation. For PCR analysis, GoTaq^{TM} G2 Green Master Mix (Promega, U.S.A.) was used in 10 µL PCR reaction, including 2 µL of DNA template, 5 µL of 2x Master Mix, 1 µL of each primer (10 nM), and 1 µL of ddH_2O. The PCR reactions were performed as follows: denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 63 °C for 45 s, and 72 °C for 1 min, with a final elongation step of 72 °C for 7 min. The PCR products were analyzed by size on 2 % agarose gel electrophoresis for 60 min at 90 V in TBE buffer with a 100 bp DNA ladder.

3.9 Genotyping

The 159 germplasm rice varieties from Yongbin et al. (2023), including 47 cold-tolerant varieties and 112 cold-sensitive varieties, were used to validate the markers. The seeds were planted in the field for two weeks under natural conditions. Leaves of seedlings in each variety were collected and extracted for DNA using the CTAB

method. The quality of the extracted DNA was estimated using a nanodrop and kept at -20 °C until use for genotyping. The DNA of 159 rice varieties was genotyped with both specific markers, following the conditions mentioned above. The association between markers and cold-tolerant traits scores was tested using simple regression analyses, conducted using the linear regression procedure in SPSS at the 0.01 level of significance.

CHAPTER 4
RESULTS

4.1 Phenotypic differences under cold stress

When exposed to cold for 24 hours, cold-sensitive varieties RD31 and SPR90, along with moderately tolerant DPY, showed signs of leaf rolling and wilting. This contrasted explicitly with the cold-tolerant varieties NB and B30, which showed minimal difference between cold and control conditions (Figure 4.1a). Extending the cold stress to 72 hours revealed a clear distinction. NB and B30 varieties displayed minimal damage compared to their controls, while DPY, RD31, and SPR90 suffered yellowing and wilting under cold stress. After recovering for a week after exposure to cold stress for 72 hours, NB and B30 seedlings grew slowly compared to control conditions. DPY, while showing some initial wilting on the first leaf, also exhibited new leaf growth compared to the control. In contrast, RD31 and SPR90 seedlings exposed to cold stress displayed yellowing, wilting, and ultimately death in 14 days (Figure 4.1b). This distinct difference was reflected in survival rates: NB, B30, and DPY had 90 %, 80 %, and 80 % survival rates, respectively, whereas RD31 and SPR90 died completely.

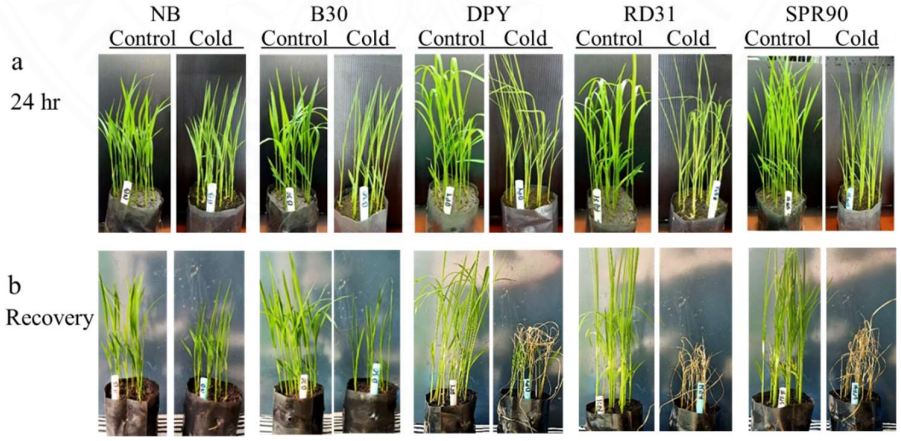


Figure 4.1 Effects of cold stress (6-8 °C) on rice seedlings. **a**) Control seedlings and seedlings exposed to cold stress for 24 hours. **b**) Seedling recovery under natural conditions for 14 days.

4.2 RNA sequencing results

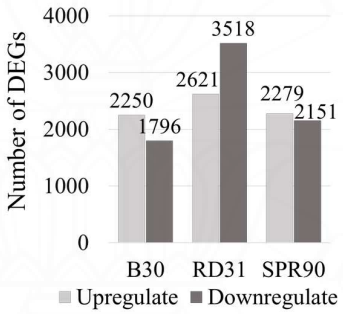
RNA sequencing was performed on six RNA samples: B30 control, B30 cold stress, RD31 control, RD31 cold stress, SPR90 control, and SPR90 cold stress. These samples were isolated from rice seedlings. The RNA sequencing generated a high number of reads, ranging from 42 to 53 million per sample. The error rate within these reads was very low, at only 0.01 %. Following quality control (QC) procedures, low-quality reads, adapter sequences, and N-containing reads (uncertain nucleotides) were removed. This filtering resulted in a clean read rate exceeding 94 % for all samples. After QC, the number of clean reads ranged from 39 to 50 million for the different samples. Alignment analysis revealed a high mapping rate. The percentage of reads that mapped to the reference genome ranged from 82.88 % to 90.62 % for total mapped reads, 81.68 % to 89.32 % for uniquely mapped reads, and 1.06 % to 1.83 % for multiply mapped reads. More details on the RNA sequencing results can be found in Table 4.1.

Table 4.1 Data quality control summary and total number of reads mapped to the reference genome.

Items	B30 control	B30 cold stress	RD31 control	RD31 cold stress	SPR90 control	SPR90 cold stress
Raw reads	49,351,808	46,428,052	51,983,714	48,835,716	42,670,952	53,045,264
Clean reads	46,691,222	43,953,670	49,108,818	46,037,726	39,961,024	50,181,698
Error rate (%)	0.01	0.01	0.01	0.01	0.01	0.01
Q20 (%)	97.96	97.78	98.17	98.02	98.12	97.92
Q30 (%)	94.66	94.31	95.12	94.85	95.01	94.58
GC content (%)	54.16	52.97	53.75	51.98	53.87	53.51
Total reads	46,691,222	43,953,670	49,108,818	46,037,726	39,961,024	50,181,698
Total mapped	42,311,067 (90.62%)	39,124,701 (89.01%)	41,399,378 (84.3%)	38,597,595 (83.84%)	33,118,218 (82.88%)	42,250,377 (84.19%)
Multiple mapped	604910 (1.30%)	464,354 (1.06%)	537,542 (1.09%)	627,951 (1.36%)	478110 (1.20)	918,019 (1.83%)
Uniquely mapped	41,706,157 (89.32%)	38,660,347 (87.96%)	40,861,836 (83.21%)	37,969,644 (82.48%)	32,640,108 (81.68%)	41,332,358 (82.37%)
Non-splice reads	28,139,859 (60.27%)	26,878,953 (61.15%)	28,411,420 (57.85%)	27,966,638 (60.75%)	22,765,671 (56.97%)	27,302,503 (54.41%)
Splice reads	13,566,298 (29.06%)	11,781,394 (26.8%)	12,450,416 (25.35%)	10,003,006 (21.73%)	9,874,437 (24.71%)	14,029,855 (27.96%)
Exons matching	94.70%	93.70%	96.70%	95.60%	96.20%	92.80%
Introns matching	1.80%	2.40%	1.20%	1.20%	1.50%	1.90%
Intergenics matching	3.50%	3.90%	2.10%	3.10%	2.40%	5.30%

4.3 Identification of differentially expressed genes (DEGs) response to cold stress in contrasting rice cultivars

To identify DEGs between cold stress and control of the three varieties, the RPKM (Reads Per Kilobase per Million reads) method was applied to normalize and quantify the gene's expression level (Mortazavi et al., 2008). Three varieties were analyzed for differential gene expression by comparing the RNA sequencing results of cold stress and control libraries in each variety. The threshold for significant differential gene expression was set at 0.005 corrected P-value and 1 log2 (fold change). The total number of DEGs in the B30, RD31, and SPR90 varieties was 4046, 6139, and 4430, respectively. The overall distribution of DEGs in each variety is presented in Figture 4.2.



Figture 4.2 The summary of total DEGs that were significantly enriched in all three groups (P-value < 0.05)

A Venn diagram analysis unveiled distinct sets of DEGs among the cold-tolerant B30 variety and the cold-sensitive RD31 and SPR90 varieties (Figure 4.3). Notably, the B30 variety has unique 748 DEGs, potentially playing a critical role in its cold tolerance mechanisms. RD31 and SPR90 also displayed unique DEGs, 2040 and 728, respectively. Table 4.2 lists the top DEGs that are unique in the B30 variety with high enrichment and read counts. Many of these highly enriched genes have unknown or hypothetical protein functions. Examples include Os06g0586000, Os01g0860450, Os08g0559800, *Os12g0230133*, *Os11g0547800*, *Os09g0550400*, *Os01g0727700*,

Os09g0246300, and *Os11g0707100*. However, the table also reveals enrichment of genes with known functions, including UDP-glucuronosyltransferases (*Os01g0175850* and *Os01g0597800*) and laccases (*Os01g0842500* and *Os05g0458600*). The analysis also identified several stress-responsive genes with high enrichment, including *OsGSTU30*, *IGPS*, *OsNCED1*, *ONAC045*, *Os01g0160800*, *Os09g0445600*, *Os12g0242100*, *Os09g0361200*, *Os08g0112300*, *ONAC045*, *SnRK2*, *OsFH10*, *HSP101*, *ISA1*, *OsFER1*, *OsProT*, *OsAP25*, *OsPAP7*, *OsEXPB11*, *OsLTP1.9*, *OsLAC*, *PFL*, and *OsLTP1.16*.

The analysis of the B30 variety's unique DEG revealed the upregulation of genes associated with abiotic stress responses and tolerance mechanisms. These findings suggest that B30 rice employs various strategies to cope with cold stress. A prominent group of upregulated genes encode transcription factors. These include *WRKYs* (*OsWRKY60*, *OsWRKY46*, and *OsWRKY15*), *NACs* (*ONAC045*, *NAC122*, and *NAC2*), and *b-ZIPs* (*OsbZIP80*, *MODD*, *OsbZIP01*, *BZIP23*, *BZIP12*, and *OsbZIP60*). Additionally, heat stress transcription factors (*HSP101* and *HSfC2b*) and others (*ATG6B* and *MODD*) were also upregulated. These TFs likely play a crucial role in activating cold stress response pathways in B30 rice. Another set of upregulated genes is involved in maintaining membrane fluidity, which is essential for proper cellular function under cold stress. This group includes calcium transporters (*OsCAX2* and *OsCAX3*), calcium-transporting ATPases (*OsACA4* and *OsACA5*), CBL-interacting protein kinases (*OsCIPK16* and *OsSOS2b*), and phospholipases (*OsPLDzeta2*). These genes likely help B30 rice cells maintain membrane integrity and function during cold exposure. The analysis identified several other important abiotic stress response genes showing upregulation. These include DREB regulons (*OsERF#007* and *OsLG3*), zinc fingers (*GATA12*, *Os03g0839000*, *Os12g0480000*, *Os04g0679800*, *Os01g0893400*, and *Os10g0500600*), ethylene response factors (*OsERF#087*, *OsERF#048*, *OS-ERS2*, *OsERF#007*, *OsLG3*, and *OsERF#050*), WD40 repeat domain-containing proteins (*OsWDR5b*, *OsWD40-112*, *OsWD40-176*, and *OsWD40-128*), receptor-like cytoplasmic kinases (*OsRLCK135*, *OsRLCK215*, *OsRLCK315*, *OsRLCK55*, and *OsRLCK28*), and bHLH domain-containing proteins (*OsbHLH150*, *OsbHLH151*, *OsbHLH061*, and *OsbHLH138*). Intriguingly, some established stress response genes, like *CIPK15* and *OsSGD1*, were downregulated. Similarly, several stress-related genes

exhibited downregulation, including Mybs (*Os05g0206200* and *Os11g0128500*), zinc fingers (*OsIDD6*, *Os11g0145400*, *Os02g0798200*, *Os02g0807700*, *Os06g0166200*, *Os04g0690100*, and *Os05g0164200*), and specific laccases (*Lac*, *OsLAC14*, *OsLAC5*, and *OsLAC*).

The cold-sensitive rice varieties, RD31 and SPR90, displayed a co-expression of 1123 DEGs, which is distinct from the cold-tolerant B30 variety (Figure 4.3). These unique DEGs likely play a crucial role in the mechanisms underlying their cold sensitivity. Interestingly, several DEGs in these cold-sensitive varieties encode proteins involved in abiotic stress responses, despite being upregulated. These included *OsUBC13*, *SAP16*, *MID1*, and *MAIF1*. Another notable finding was the upregulation of a significant number of ethylene response factors (ERFs) in the cold-sensitive varieties. These ERFs include *OsERF1*, *OsERF121*, *OsERF065*, *OsERF020*, *DERF5*, *OsERF069*, and *OsERF064*. In contrast, downregulation was observed in various stress-response genes (*ONAC003*, *SAPK3*, and *SNAC1*), plasma membrane intrinsic proteins (*OsPIP2* and *OsPIP1*), UDP-glucosyltransferase family proteins (*UGT709A4*, and *UDP-GT*), and MYB transcription factors (*OsMYB42/85*, *OsMYB55/61*, *OsMYB91*, *OsMYB55*, *OsMYB1R1*, *OsMYB86-L1*, and *OsMYBS1*). The downregulation of these genes might contribute to the impaired ability of RD31 and SPR90 to cope with cold stress.

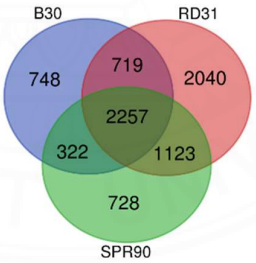


Figure 4.3 Venn diagram of DEGs showing numbers of overlap and unique DEGs between all three varieties.

Table 4.2 50 DEGs represent high enrichment and a more read count.

Gene ID	log2.Fold change.	Gene name/Gene description
Os01g0160800	8.65	Similar to Protein synthesis inhibitor II
Os01g0175850	8.45	UDP-glucuronosyl/UDP-glucosyltransferase domain containing protein.
Os09g0445600	8.40	Similar to oxidoreductase/ transition metal ion binding protein.
Os10g0529500	7.60	*OsGSTU30*: Glutathione s-transferase 30
Os11g0601950	7.56	Similar to cDNA clone:002-114-B06
Os09g0255400	6.81	*IGPS*: Indole-3-glycerol phosphate synthase
Os06g0586000	6.78	Conserved hypothetical protein.
Os08g0307601	6.70	Similar to cDNA clone:002-114-B06
Os03g0645900	6.70	*OsNCED1*: Carotenoid oxygenase family protein
Os12g0242100	6.55	Similar to Glycine-rich cell wall structural protein 1 precursor.
Os01g0860450	6.33	Hypothetical protein.
Os08g0559800	6.29	Hypothetical conserved gene.
Os12g0230133	6.25	Conserved hypothetical protein.
Os11g0547800	6.24	Conserved hypothetical protein.
Os01g0597800	6.21	UDP-glucuronosyl/UDP-glucosyltransferase family protein.
Os09g0550400	6.16	Hypothetical protein.
Os09g0361200	6.05	Similar to hypersensitive-induced response protein.
Os08g0112300	6.01	Transferase domain containing protein.
Os03g0797500	5.90	Similar to Indole-3-glycerol phosphate lyase (Fragment).
Os11g0127600	5.53	*ONAC045*: NAC domain-containing protein 45
Os04g0629300	5.32	*SnRK2*: Dead-like helicase, N-terminal domain containing protein.
Os02g0161100	4.48	*OsFH10*: Actin-binding FH2 domain containing protein.
Os03g0792800	4.25	Beta-1,3-glucanase-like protein.
Os01g0727700	4.21	Hypothetical conserved gene.
Os05g0519700	4.14	*HSP101*: Heat shock protein 101
Os08g0520900	3.72	*ISA1*: Isoamylase 1
Os11g0106700	3.06	*OsFER1*: Ferritin 1
Os03g0644400	2.78	*OsProT*: proline transporter, proline transporter 2
Os04g0175600	-4.39	*OsCOMTL5*: Caffeic acid 3-o-methyltransferase like 5
Os07g0658600	-4.63	Similar to Nucleoid DNA-binding-like protein.

Table 4.2 50 DEGs represent high enrichment and a more read count. (continue)

Gene ID	log2.Fold change.	Gene name/Gene description
Os04g0468000	-5.39	Similar to *OSIGBa0128P10.7* protein.
Os02g0308400	-5.62	*OsFLA19*: Fasciclin-like arabinogalactan protein 19
Os09g0246300	-5.75	Hypothetical protein
Os01g0842500	-5.85	*OsLAC5*: Laccase 5
Os11g0210100	-5.88	*prx132*: Class III peroxidase 132, 133
Os10g0474800	-5.98	Similar to predicted protein.
Os10g0409400	-6.28	*RD22*: Polygalacturonase isoenzyme 1 beta subunit
Os04g0137100	-6.43	Similar to *OSIGBa0147B06.7* protein.
Os03g0186900	-6.71	*OsAP25*: Aspartic protease 25
Os11g0549620	-6.82	*OsPAP7*: Purple acid phosphatase 7
Os02g0658800	-7.11	*OsEXPB11*: Beta-expansin 11
Os10g0150700	-7.28	Protein of unknown function DUF1210 family protein.
Os10g0150600	-7.76	Protein of unknown function DUF1210 family protein.
Os11g0707100	-7.94	Hypothetical conserved gene.
Os10g0150400	-8.05	Protein of unknown function DUF1210 family protein.
Os10g0150800	-8.40	Protein of unknown function DUF1210 family protein.
Os11g0115100	-8.46	*OsLTP1.9*: Lipid transfer protein 1.9
Os05g0458600	-8.52	*OsLAC*: Laccase 15
Os06g0553200	-8.93	*PFL*: Protodermal factor like
Os12g0114800	-10.22	*OsLTP1.16*: Lipid transfer protein 1.16

4.4 Enrichment analysis of unique DEGs in cold-tolerant variety

To understand the biological functions and pathways potentially associated with cold tolerance in B30 rice, researchers analyzed the 748 DEGs specific to this variety using the online tool ShinyGO 0.77. The GO term analysis revealed 86 annotations categorized into three main categories: biological process (22 terms), cellular component (37 terms), and molecular function (27 terms). Interestingly, enriched GO terms included "organelle membrane," "chloroplast," "plastid," and "metal ion transport" (Figure 4.4a). The analysis also identified six enriched KEGG pathways associated with B30's DEGs (Figure 4.4b). These pathways included "metabolic pathways," "biosynthesis of secondary metabolites," "nitrogen metabolism," "fatty acid degradation," "cutin, suberine, and wax biosynthesis," and "inositol phosphate

metabolism." Notably, pathways like "metabolic pathways," "secondary metabolite biosynthesis," and "nitrogen metabolism" showed significant enrichment, suggesting their potential importance for B30's response to cold stress.

Figure 4.4 Enrichment analysis focused on the 748 DEGs uniquely expressed in the cold-tolerant B30 variety. **a**) The 30 most enriched GO terms are presented in two categories: biological processes and cellular components. **b**) This figure depicts the KEGG pathway enrichment analysis based on the FDR. The X-axis represents the specific metabolic pathway, and the Y-axis represents the fold enrichment of DEGs associated with that pathway. Asterisks indicate pathways with statistically significant enrichment (FDR-adjusted p-value ≤ 0.05). The size of each circle reflects the number of DEGs associated with that pathway. The most significantly enriched pathways are highlighted in red based on their -log10 (FDR) values.

4.5 Validation of RNA sequencing data results

To verify the accuracy of the RNA sequencing data, twelve randomly chosen DEGs (differentially expressed genes) from the cold-tolerant B30 variety were analyzed using semi-RT PCR (reverse transcription polymerase chain reaction). These DEGs included both upregulated and downregulated genes in B30 compared to the other varieties. Seedling samples from all five rice varieties were used in the analysis.

The results of the semi-RT PCR analysis of twelve DEGs were mostly consistent with the RNA sequencing data (Figure 4.5). *GAPDH* was used as the reference gene for normalizing gene expression levels. Based on these results, six genes (*OsFH10*, *ONAC045*, *SnRK2*, *OsISA1*, *OsFER1*, and *OsProT*) were chosen for further investigation due to their potential role in B30's cold tolerance.

We focus on the expression patterns of six particularly interesting genes: *OsFH10*, *ONAC045*, *SnRK2*, *OsISA1*, *OsFER1*, and *OsProT*. These genes exhibited distinct expression patterns between cold-tolerant and cold-sensitive rice varieties (Figure 4.5). The *OsFH10* and *SnRK2* genes showed significant upregulation under cold stress only in cold-tolerant varieties. In contrast, they were undetectable in cold-sensitive varieties under both cold and control conditions. The *OsISA1* and *ONAC045* genes displayed a stronger upregulation response in cold-tolerant varieties compared to the minimal upregulation observed in cold-sensitive varieties under cold stress. *OsProT* and *OsFER1* genes exhibited marked upregulation in cold-tolerant varieties under cold stress, while no change in expression was detected in cold-sensitive varieties under both conditions. These findings suggest that *OsFH10*, *ONAC045*, *SnRK2*, *OsISA1*, *OsFER1*, and *OsProT* may play a crucial role in conferring cold tolerance in rice seedlings. Subsequently, the study explored the molecular mechanisms governing the expression of these six genes, including promoter sequence analysis, DNA methylation profiling, alternative splicing (AS), and marker development. Details regarding these genes are presented in Table 4.4.

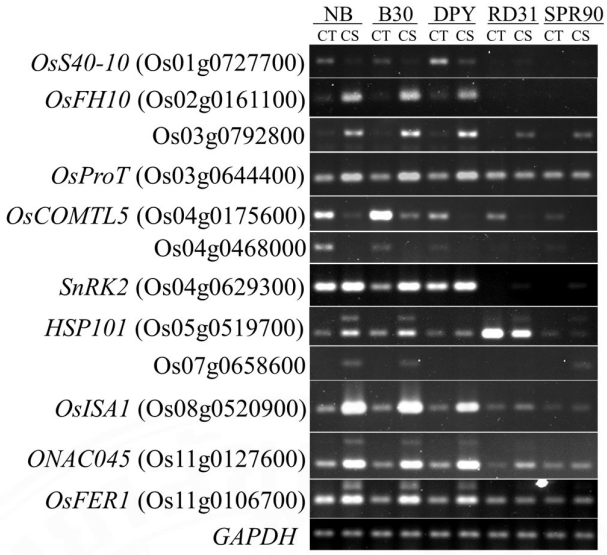


Figure 4.5 Validation of RNA sequencing results by semi-RT PCR. Twelve DEGs, including *OsS40-10*, *OsFH10*, *Os03g0792800*, *OsProT*, *OsCOMTL5*, *Os04g0468000*, *SnRK2*, *HSP101*, *Os07g0658600*, *OsISA1*, *ONAC045* and *OsFER1* were randomly selected for expression analysis by semi-RT PCR. Control (CT): 30±3 °C. Cold stress (CS): 6-8 °C

Table 4.3 Primer sequences.

Experiment	Gene	Forward sequences (5'–>3')	Reverse sequences (5'–>3')
RT-PCR	*Os02g0161100*; *FH10*	GGCAAATCTGCGACACGAAT	AACAGGCGAAAACCGTCATC
	Os05g0519700; *HSP101*	AGAACCTGATGCTCACCGAG	GGACCGAGGAAGAGAAACGA
	Os03g0792800	ACGTCTCCCGCTACAACTTC	AGTCGTATACGTCGGCGTTG
	Os07g0658600	GCTTCAGCCTCTCCTACGC	CCCGTACATGTCCTTGGTCTG
	Os01g0727700; *OsS40-10*	CCGTTCACGGACCCGAT	AGGACGGAGTCGCGCA
	Os04g0175600; *OsCOMTL5*	AACCTCCCCGGAGACGATAC	CGGTTCGTCCCCATGTACTC
	Os04g0468000	CAGACCGAGATTCTTATCTGGATTC	AGACTGCACTCCTGCTCGG
	Os11g0127600; *ONAC045*	CCTCAACAAGTGCGAGCCAT	TTGGGAGCTCTCCCCATGTA
	Os04g0629300; *SnRK2*	CGAGATGCCTACATCTGCAA	TGACTGATTTGCTTCAGGGCT
	Os08g0520900; *OsISA1*	CGAGATCTACGTGGCCTTCAA	AGTAGCTGAGCATGGGGTAGA
	Os11g0106700; *OsFER1*	CGCTAGGCAAAAGTTCGTCG	GGTGTGACGATGGACTGGAG
	Os03g0644400; *OsProT*	TCAGAGGAGGCTACCTGACC	CCAACAACGTTCAGCCAGTG
AS analysis	FH10-IsF1	GGCGCCATCTTTCTTCTGGA	TGGATGGCCTAACCCCATGA
	FH10-IsF2	TGGCCTGGCATGACATCAAA	TTCCATGGCGAGTCTTGCTT
	NAC045-IsF	TCGACCTCAACAAGTGCGAG	CGTCGTACTTGCACCGTAGT
	SnRK2-IsF	TCGGGCTGGATGACGATAAC	AAGTTGACGAAGGCGATGGT
	ISA1-IsF1	AATTTCGCCGTCTACTCCGC	GCCCCAATGTAAGTCCCTGG
	ISA1-IsF2	GGGAGGCCTCTATCAAGTAGG	TTTTGACCGACAAACCTGCAA
	FER1-IsF	CGCTAGGCAAAAGTTCGTCG	GGTGTGACGATGGACTGGAG
	ProT-IsF1	GGATACGGCGCACCAGATTA	TCCCAACCAAATCCTGAGAGC
	ProT-IsF2	GCTCTCAGGATTTGGTTGGGA	CCAACAACGTTCAGCCAGTG

Table 4.3 Primer sequences. (continue)

Experiment	Gene	Forward sequences (5'–>3')	Reverse sequences (5'–>3')
MSP	*OsFH10*; B primer	GTAGTTATATTTTTGATGTTTTTAGAGTA	ACCACACACTAACTTTTATACCAC
	OsFH10; M primer	GTGGTTTATAAGTCGGTGTGTAGC	ACCTTTATCTATTCGACGAAAACG
	OsFH10; U primer	GTGGTTTATAAGTTGGTGTGTAGTG	CCTTTATCTATTCAACAAAAACACA
Marker Development	*OsProT*	CACTCCTCTCCCCTCCCC	CTCGTTCGTTGACTCCCCTC
	ONAC045	ATAATAATAATTCGACTATAAGATAGCTG	TATACAAGTTGAGCTTAATTACATCAAT
		AGCACTATACATGAAAATAAAGAAGTC	GAGTTTATTTATAAAGCTTTGAGACC

Table 4.4 Descriptions of six interesting genes are expected to be associated with cold tolerance in rice seedlings.

ID genes	Gene name	Description
Os02g0161100	*OsFH10*	Actin-binding FH2 domain containing protein
Os11g0127600	*ONAC045*	No apical meristem (NAM) protein domain containing protein
Os04g0629300	*SnRK2*	Similar to H0303G06.18 protein
Os08g0520900	*OsISA1*	Starch-debranching enzyme, Isoamylase 1, Amylopectin biosynthesis
Os11g0106700	*OsFER1*	Similar to Ferritin 1, chloroplast precursor
Os03g0644400	*OsProT*	Amino acid permease

4.6 *OsFH10*

According to validation of gene expression by real-time RT-PCR, expression of *OsFH10* genes was much higher upregulated in the cold-tolerant varieties B30 under cold stress than those in their control condition. However, this gene was able to be detected at extremely low levels in sensitive varieties RD31 and SPR90, but at much lower levels than those in B30 (Figure 4.6a).

We investigated DNA methylation as a potential factor regulating expression. Because *OsFH10* expression differed significantly between cold-tolerant and sensitive varieties, DNA methylation may be a factor regulating expression. Methylation in the promoter region, which can influence gene expression, was analyzed using methylation-specific PCR (MSP). The MSP results revealed distinct methylation patterns between cold-tolerant and cold-sensitive varieties (Figure 4.6b). In cold-sensitive varieties (RD31 and SPR90), methylated alleles were amplified under both cold stress and control conditions. Conversely, no methylation allele was detected in the promoter region of cold-tolerant varieties (NB, B30, and DPY) under both conditions. Further, unmethylated alleles were amplified in all cold-tolerant varieties under both cold and control conditions, while no amplification was detected in cold-sensitive varieties. In summary, cold-tolerant varieties have no methylation (0 %) in the promoter region of this gene, allowing for its expression. Conversely, cold-sensitive varieties exhibit 100 % methylation in the promoter region.

Analysis of *OsFH10* revealed the presence of two isoforms: normal and intron2 retention (IR2) isoforms. The primer locations and corresponding gene structures for the *OsFH10* gene are presented in Figure 4.6c. PCR product of FH10-IsF1 displayed AS with two isoforms: a normal isoform (813 bp) containing exons1 - exons3 and an IR2 isoform (890 bp) due to retention of intron2. The IR2 isoform, resulting from IR at intron2, exhibits a larger size compared to the normal isoform due to the retained 77 bp of intron2. This isoform was specifically upregulated in the three cold-tolerant varieties only under cold stress conditions, with no observed in cold-sensitive varieties. On the other hand, FH10-IsF2 exhibited only a normal isoform (770 bp) encompassing exon3 - exon6. This isoform was upregulated in cold-tolerant varieties under cold stress but displayed slight downregulation in all cold-sensitive varieties (Figure 4.6d).

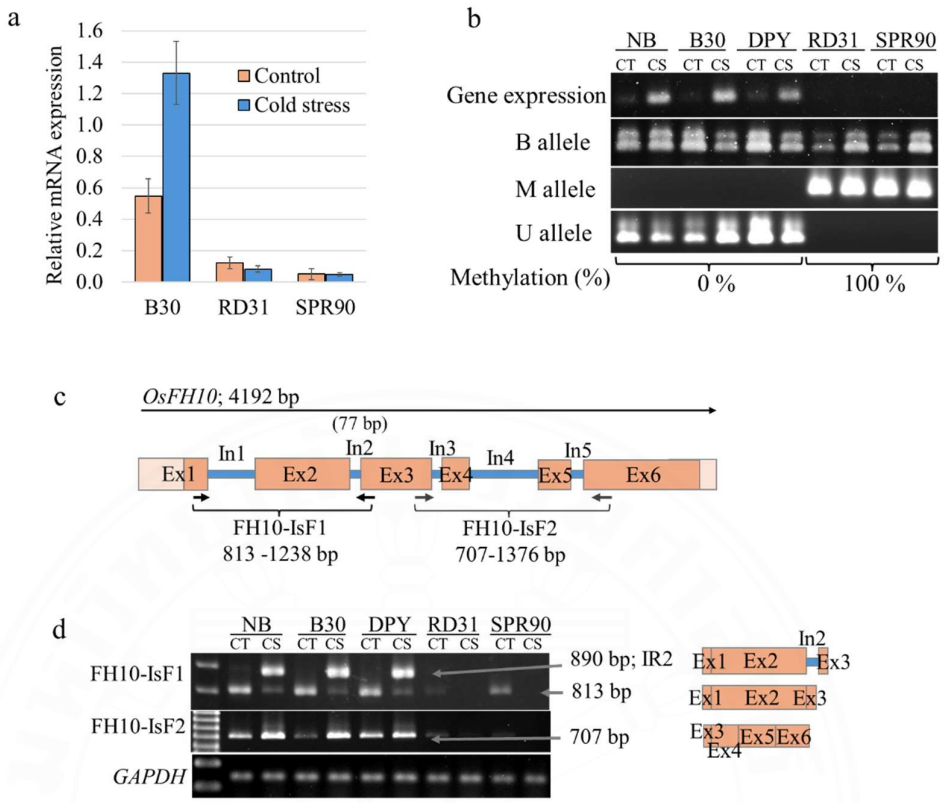


Figure 4.6 *OsFH10* gene expression pattern analysis and DNA methylation. **a**) Expression analysis of *OsFH10* by real-time RT-PCR in cold tolerant (B30) and cold sensitive (RD31 and SPR90) varieties. The expression level has been normalized with *GAPDH*. Data shown were mean ± SD and ΔΔCt levels (n = 3). **b**) Methylation specific PCR analysis of the *OsFH10* gene (B, bisulfite; M, methylated; U, unmethylated). **c**) The gene structures show primer locations for alternative splicing. **d**) Alternative splicing events responded to the cold stress by semi-RT PCR in five seedling varieties.

4.7 *OsISA1*

Consistent with the previous findings, the results revealed a significantly higher upregulation of *OsISA1* in the cold-tolerant B30 variety under cold stress. Conversely, expression levels of *OsISA1* remained very low in the cold-sensitive varieties (RD31 and SPR90) under cold stress (Figure 4.7a).

Analysis of *OsISA1* expression revealed the occurrence of cold stress-induced AS events specifically in cold-tolerant rice varieties (B30, NB, and DPY).

These varieties displayed PCR products of varying sizes under cold stress conditions, while cold-sensitive varieties exhibited no such variation. The primer locations and corresponding gene structures for the *OsISA1* gene are presented in Figure 4.7b. Two isoforms were identified from ISA1-IsF1: normal (476 bp) and IR1+IR2+IR3 (1069 bp) isoforms. The normal isoform comprises four exons (exon1-exon4) and showed no significant difference in expression level between cold-tolerant and cold-sensitive varieties under both control and cold-stress conditions. Conversely, the IR1+IR2+IR3 isoform results from the retention of introns 1, 2, and 3 (122 bp, 104 bp, and 356 bp, respectively). This isoform was exclusively upregulated in cold-tolerant varieties upon cold-stress exposure. Similar to the isoform from ISA1-IsF1, the PCR product of ISA1-IsF2 generated two isoforms: normal (344 bp) and IR12+IR13 (1268 bp) isoforms. The normal isoform of ISA1-IsF2 displayed no difference in expression between cold-tolerant and cold-sensitive varieties under both conditions. The IR12+IR13 isoform results from the retention of introns 12 and 13 (488 bp and 436 bp, respectively). This isoform was also exclusively upregulated in cold-tolerant varieties upon exposure to cold stress. Interestingly, under cold stress conditions, both normal isoforms of *OsISA1* (IsF1 and IsF2) showed no difference in expression between cold-tolerant and cold-sensitive varieties. However, combined intron retention events (IR1+IR2+IR3+IR12+ IR13) were specifically upregulated in the three cold-tolerant varieties Figure 4.7c.

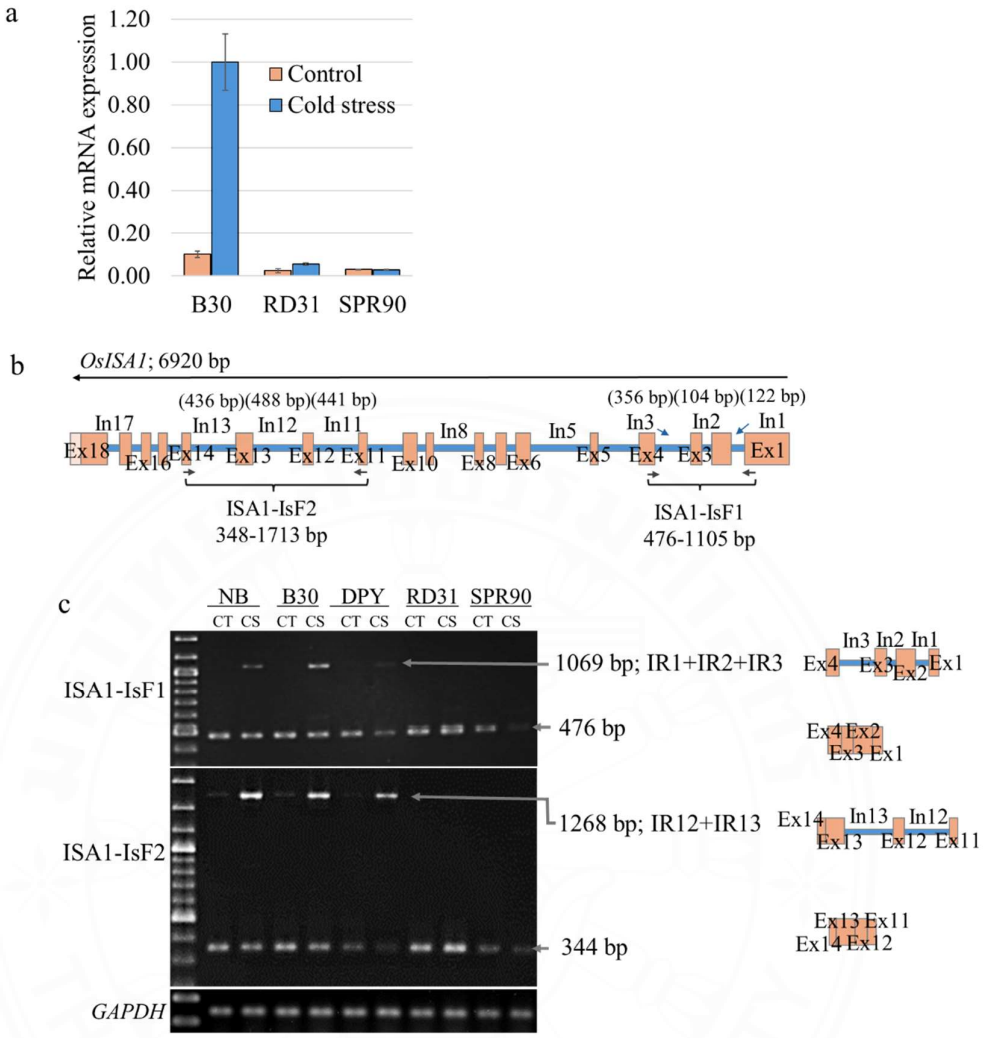


Figure 4.7 *ISA1* gene expression pattern analysis. **a**) Real-time RT-PCR analysis of *ISA1* in cold tolerant (B30) and cold sensitive (RD31 and SPR90). The expression level was normalized with *GAPDH*. Data shown mean ± SD and ΔΔCt levels (n = 3). **b**) The gene structures show primer locations for alternative splicing. **c**) Alternative splicing events responded to the cold stress by semi-RT PCR in the five seedling varieties.

4.8 *ONAC045*

Consistent with the results from semi RT-PCR, qRT-PCR analysis revealed significantly higher upregulation of *ONAC045* gene expression in the cold-tolerant B30 variety under cold stress. Conversely, RD31 and SPR90 displayed lower expression,

with no significant difference observed between the control and cold stress treatments (Figure 4.8a).

ONAC045 expression analysis under cold stress revealed the generation of four AS isoforms exclusively in cold-tolerant varieties. The primer locations and corresponding gene structures for the *ONAC045* gene are presented in Figure 4.8b. These isoforms included the normal form (644 bp) and three isoforms resulting from IR events: IR1 (809 bp), IR2 (776 bp) and IR1+IR2 (941 bp). The normal isoform contains three exons (exon1 - exon3) and is upregulated in all cold-tolerant varieties under cold stress, but cold-sensitive varieties displayed similar expression levels under both control and cold-stress conditions. IR1 isoform results from retaining 165 bp of intron1, larger than the normal isoform. Because of their similar size, the DNA bands could be IR2 isoform resulting from intron2 retention. Finally, the IR1+IR2 isoform displayed retention of both introns (intron1 and intron2) and was larger by 297 bp compared to the normal form. IR1, IR2 and IR1+IR2 were upregulated only in cold-tolerant varieties under stress, not present in cold-sensitive varieties. In contrast, cold-sensitive varieties expressed only the normal isoform with no change under cold stress (Figure 4.8c).

Polymorphisms within promoter sequences can potentially influence gene expression. Two SNPs were identified in the promoter region of *ONAC045*, differentiating cold-tolerant varieties NB and B30 from cold-sensitive varieties 9311, RD31, and SPR90. These SNPs are located at 59 and 135 bp upstream of the TSS. Notably, the SNPs reside near the TATA-box region but did not indicate motif elements (Figure 4.8d). These sequence variations may contribute to the observed differences in gene expression levels between cold-tolerant and cold-sensitive varieties. Furthermore, these SNPs could be used to develop potential markers for genotyping, facilitating the selection of germplasm with desirable cold tolerance traits.

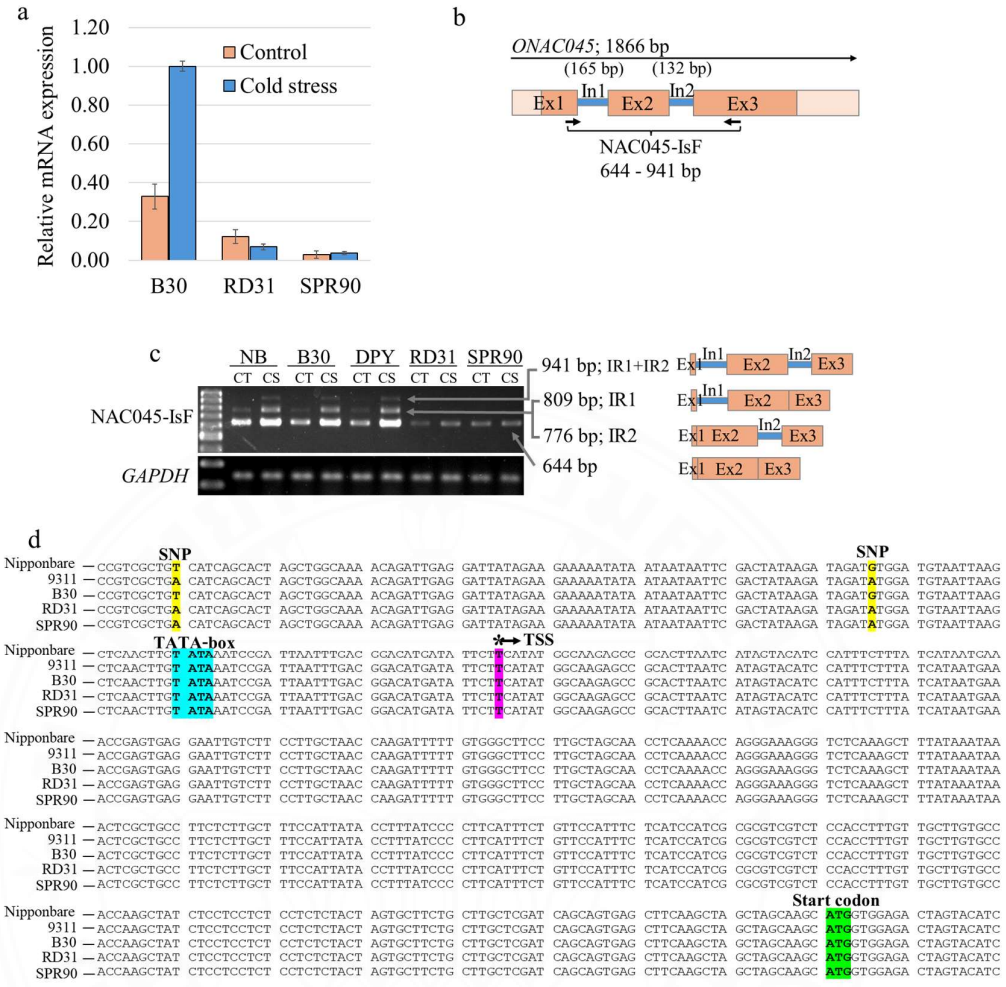


Figure 4.8 *ONAC045* gene expression pattern analysis and promoter sequence validation. **a**) Expression analysis of *ONAC045* by real-time RT-PCR in cold tolerant B30 and cold sensitive RD31 and SPR90 varieties. The expression level has been normalized with *GAPDH*. Data shown mean ± SD and ΔΔCt levels (n = 3). **b**) The gene structures show primer locations for alternative splicing. **c**) Alternative splicing events responded to the cold stress by semi-RT PCR in five seedling varieties. **d**) The SNPs in the promoter regions of the *ONAC045* genes. The sequences from B30, RD31, and SPR90 were compared to those in Nipponbare and 9311.

4.9 *SnRK2*

According to validation of gene expression using real-time RT-PCR, the expressions of this gene were high-expressed under both conditions in cold-tolerant B30 and were few upregulated under cold stress in cold-sensitive RD31 and SPR90 (Figure 4.9b a).

The effect of cold stress on AS in *SnRK2* was investigated by semi-RT PCR. The primer locations and corresponding gene structures for the *SnRK2* gene are presented in Figure 4.9b. Notably, only the cold-tolerant B30, NB, and DPY varieties produced PCR products of varying sizes under cold stress, whereas sensitive RD31 and SPR90 varieties produced only one size of PCR product (Figure 4.9c). This suggests the presence of distinct AS events specifically within the *SnRK2* transcripts of cold-tolerant varieties under cold stress conditions. Four isoforms show exons skipped, leading to transcript diversity. Based on the transcript size, this transcript likely corresponds to the normal, ES3, ES6, and ES5 isoforms of *OsISA1*. These isoforms were upregulated in cold-tolerant varieties under only cold stress. The normal isoform (1155 bp) represents the full-length transcript and includes exon2-exon7. The ES3 isoform (1008 bp) skips exon3, resulting in a shorter transcript compared to the normal isoform. Furthermore, cold-tolerant varieties produced a single transcript of approximately 1080-1083 bp, indicating ES6 (1080 bp) or ES5 (1083 bp) isoforms of AS activity under cold stress. The ES6 isoform arises from skipping exon6, resulting in a predicted size of 1080 bp, while the ES5 isoform arises from skipping exon5, resulting in 1083 bp.

Genetic variations in promoter sequences could potentially modulate gene expression. A SNP in the *SnRK2* promoter was identified between cold-tolerant NB and B30 varieties and cold-sensitive 9311, RD31, and SPR90 varieties. This SNP was located 19 base pairs upstream of the TSS. Analysis of SNP locations within UTRs revealed the presence of SNPs near TSS regions; however, these SNPs did not indicate motif elements (Figure 4.9d).

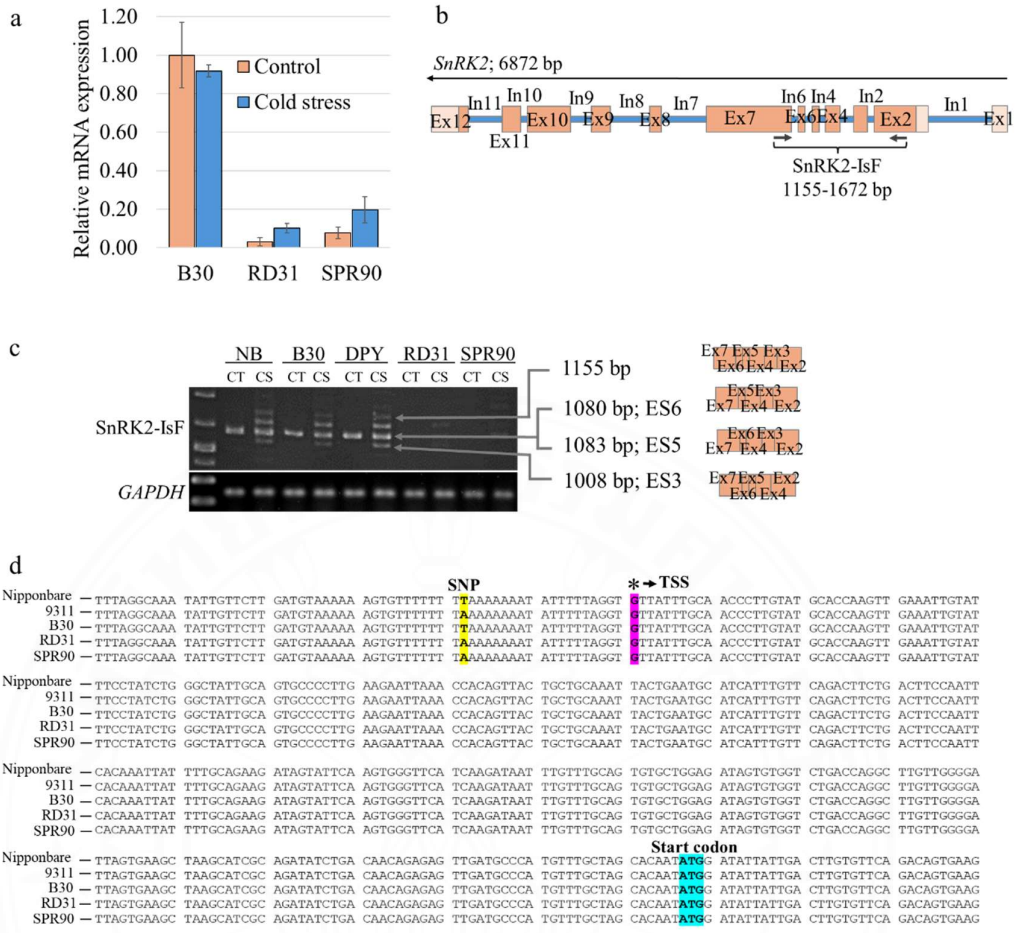


Figure 4.9 *SnRK2* gene expression pattern analysis and promoter sequence validation. **a**) Expression analysis of *SnRK2* by real-time RT-PCR in cold tolerant B30 and cold sensitive RD31 and SPR90 varieties. The expression level has been normalized with *GAPDH*. Data shown were mean ± SD and ΔΔCt levels (n = 3). **b**) The gene structures show primer locations for alternative splicing. **c**) Alternative splicing events responded to the cold stress by semi-RT PCR in five seedling varieties. **d**) Promoter sequences of the *SnRK2* genes. The sequences from B30, RD31, and SPR90 were compared to those in Nipponbare and 9311.

4.10 *OsFER1*

Consistent with the findings from semi-quantitative RT-PCR validation, real-time RT-PCR analysis revealed upregulation under cold stress in all analyzed

samples, with the highest expression observed in the cold-tolerant B30 variety (Figture 4.10a).

To investigate the impact of cold stress on AS events within the *OsFER1* gene, semi-RT-PCR was performed using a specific primer, FER1-IsF. The gene structure and primer region are shown in Figture 4.10b. Analysis of *OsFER1* revealed complex AS exclusively in cold-tolerant varieties under cold stress, whereas cold-sensitive varieties only expressed the normal isoform. Four isoforms of FER1-IsF were identified: normal, IR2, IR3, and IR2 + IR3 (intron2 and intron3 retention). The normal isoform (243 bp) comprised four exons (exon1 - exon4) and was upregulated solely in cold-tolerant varieties under stress. Conversely, cold-sensitive varieties have no difference in expression levels under control and cold-stress conditions. IR2 (323 bp) resulted from intron2 retention and was larger than the normal isoform by 80 bp. Similarly, IR3 (346 bp) exhibited retention of intron3 and was larger by 103 bp compared to the normal form. Both IR2 and IR3 were upregulated only in cold-tolerant varieties under stress, not present in cold-sensitive varieties. Finally, the IR2+IR3 isoform (426 bp) displayed retention of both introns (intron2 and intron3) and was larger by 183 bp compared to the normal form. It is mildly upregulated in cold-tolerant varieties under stress, but not detected in cold-sensitive varieties (Figture 4.10c). In conclusion, cold-tolerant varieties displayed upregulation of all four isoforms exclusively under cold stress, whereas cold-sensitive varieties only expressed the normal isoform constitutively.

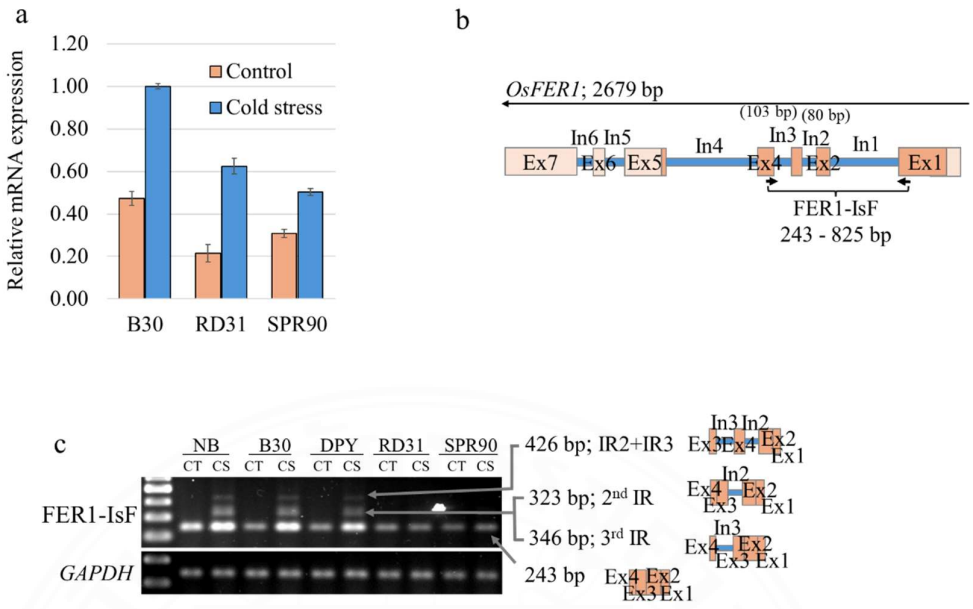


Fig 4.10 *OsFER1* gene expression pattern analysis. **a**) Real-time RT-PCR analysis of *OsFER1* in cold tolerant (B30) and cold sensitive (RD31 and SPR90). The expression level was normalized with *GAPDH*. Data shown mean ± SD and ΔΔCt levels (n = 3). **b**) The gene structures show primer locations for alternative splicing. **c**) Alternative splicing events responded to the cold stress by semi-RT PCR in the five seedling varieties. **d**) Promoter sequences of the *OsFER1* genes from B30 compared to those from RD31, and SPR90.

4.11 *OsProT*

Validation of gene expression by qRT-PCR revealed a significant upregulation of the *OsProT* gene under cold stress compared to the control condition in the cold-tolerant variety B30. Conversely, cold-sensitive varieties (RD31 and SPR90) exhibited similar expression levels for this gene under cold stress (Figure 4.11a).

OsProT expression analysis revealed cold stress-induced AS events specifically in cold-tolerant rice varieties. The primer locations and corresponding gene structures for the *OsProT* gene are presented in Figure 4.11b. These varieties exhibited PCR products of varying sizes under cold stress, while cold-sensitive varieties displayed no such variation. Two isoforms were identified from ProT-IsF1: normal (475 bp) and

IR4 (670 bp) isoforms. The normal isoform comprised four exons (exon2 - exon5) and showed no difference under both control and cold stress conditions in all varieties. Conversely, the IR4 isoform, which results from retaining 195 bp of intron4, was exclusively upregulated in cold-tolerant varieties upon cold-stress exposure. Similarly, the PCR product of ProT-IsF2 generated two isoforms: normal (770 bp) and IR7 (852 bp) isoforms. This normal isoform, comprising four exons (exon5 - exon8) shows no difference in expression levels in all varieties under both conditions. The IR7 isoform, which results from retaining 82 bp of intron7, was exclusively upregulated in cold-tolerant varieties upon cold-stress exposure. Ultimately, under cold stress, both normal isoforms (exon2 - exon8) showed no distinction in expression levels. Furthermore, combined IR events at introns4 and intron7 (IR4+IR7) were upregulated, especially in the three cold-tolerant varieties (Figure 4.11c).

Analysis of the *OsProT* promoter sequence revealed a total of 18 SNPs distributed across the gene promoter. Specifically, 18 SNPs were found located at 29, 169, 260, and 305 upstream and 5, 25, 41, 52, 128, 132, 134, 140, 167, 197, 224, 226, 232, and 244 downstream of the TSS (Figure 4.11d). Focusing on the predicted locations of SNPs within the UTRs, two SNPs were identified near the TSS. Notably, an analysis of regulatory motifs within these regions predicted the presence of a STRE element. Additionally, two deletions were discovered within the promoter region: a six-nucleotide deletion located at positions 5-10 bp upstream and a five-nucleotide deletion located at positions 44-48 bp downstream of the TSS. The deletion occurring closest to the TSS (5-10 bp upstream) is positioned within the core region of a DRE core.

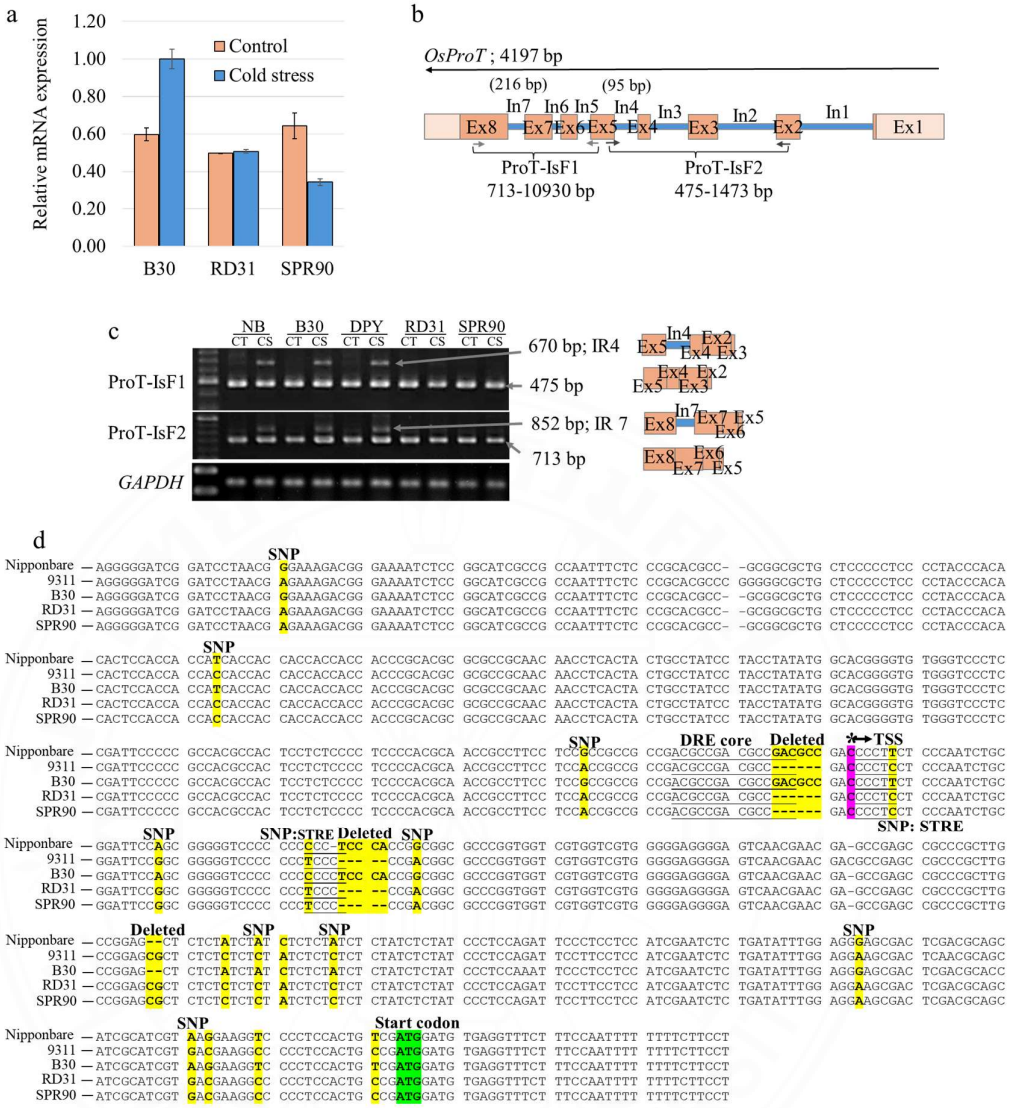


Figure 4.11 *OsProT* gene expression pattern analysis and promoter sequence validation. **a**) Expression analysis of *OsProT* by Real-time RT-PCR in cold tolerant (B30) and cold sensitive (RD31 and SPR90) varieties. The expression level was normalized with *GAPDH*. Data shown mean ± SD and ΔΔCt levels (n = 3). **b**) The gene structures show primer locations for alternative splicing. **c**) Alternative splicing events responded to the cold stress by semi-RT PCR in five seedling varieties. **d**) The SNPs in the promoter regions of *OsProT* genes. The sequences from B30, RD31, and SPR90 were compared to those in Nipponbare and 9311.

4.12 Marker development and genotyping

4.12.1 *ONAC045*

The SNP in the promoter of *ONAC045* was used to develop a DNA marker for PCR agarose gel-based genotyping by the tetra primer PCR method (Kim et al., 2016). Genotyping using agarose gel electrophoresis is a simple laboratory requirement and operation. Two primer pairs were used in a single PCR tube to amplify both mutant and normal alleles, with an internal control band (Figure 4.12.1a). The G allele (248 bp) is cold-tolerant, whereas the A allele (209 bp) is cold-sensitive. The marker had excellent amplicons showing distinct DNA patterns between tolerance and sensitive varieties using 2 % agarose gel (Figure 4.12.1b). To validate this marker, 159 rice varieties used in Yongbin et al. (2023) showing cold tolerant and sensitive phenotypes were genotyped. The results of genotyping showed that 51.06 % of tolerant varieties had the tolerance allele (24 out of 47) of this gene, while 98.21 % of sensitive varieties (111 out of 112) had the sensitive allele (Figure 4.12.1c). The tolerant allele was found in all tested groups (*japonica*, *indica*, admixture, and n/a). The tolerant allele was present in 94.74 % (18 out of 19) of tolerant *japonica*, but only in 9.52 % (2 out of 21) of tolerant *indica* (Figure 4.12.1d). A one-way analysis of variance (ANOVA) and regression analysis conducted using SPSS 16.0 software indicated a significant association ($P < 0.05$) between the allele identified by the *ONAC045* marker and the cold tolerance score of 159 rice germplasm accessions under cold stress at the seedling stage. This regression analysis has an R-squared value of 0.438, indicating that the *ONAC045* marker allele can explain 43.8 % of the variation in cold tolerance at the seedling stage in 159 rice germplasm, with a 95 % confidence level.

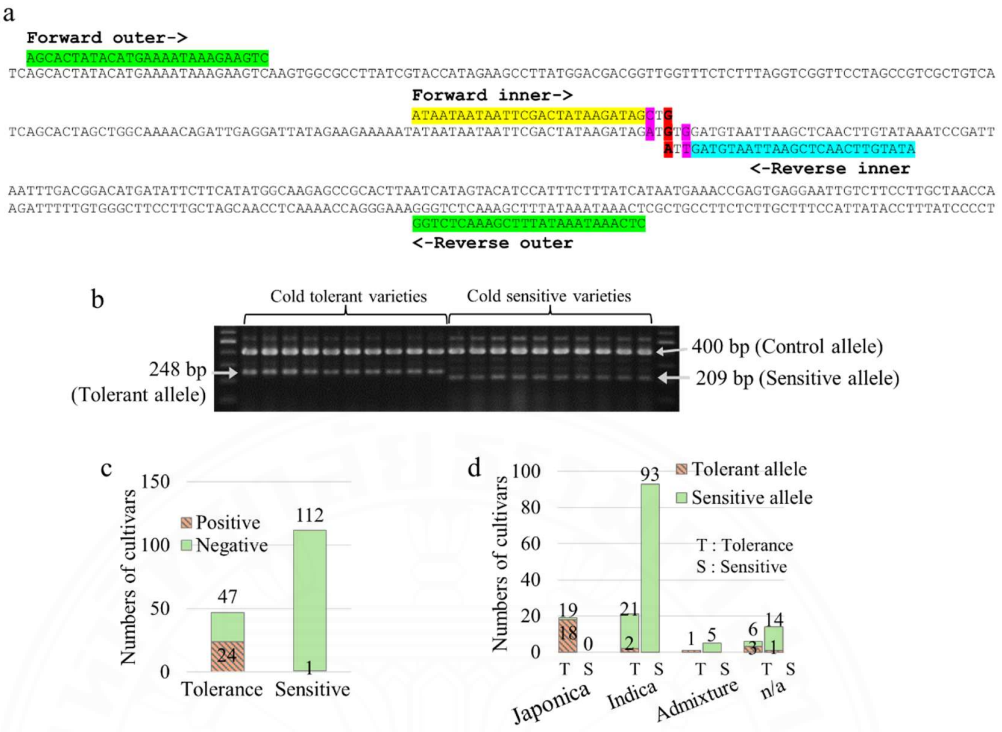


Figure 4.12.1 Development and validation of the *ONAC045* SNP marker. **a**) Diagram of tetra-primer ARMS-PCR with the G/A SNP in the *ONAC045* promoter highlighted in red. The two outer primers (green) amplify regions distantly located from the SNP, while the inner primers are designed to specifically amplify the two alleles: the forward inner primer (yellow) amplifies the G allele, and the reverse inner primer (blue) amplifies the A allele, resulting in distinguishable PCR products of different sizes on an agarose gel. **b**) Representative genotyping samples include ten cold-sensitive and ten cold-tolerant varieties. Cold-tolerant varieties exhibit a PCR product corresponding to the G allele (248 bp), whereas sensitive varieties show a product corresponding to the A allele (209 bp). **c**) The allele frequency of *ONAC045* in extremely tolerant (47) and extremely sensitive (112) rice varieties. Orange indicates the proportion of tolerant alleles, while the green bars indicate the proportion of sensitive alleles in two rice variety groups. **d**) The distribution of tolerant and sensitive alleles of *ONAC045* within each rice subspecies group: japonica (19), indica (114), admixture (6), and not available (20). Here, T denotes cold-tolerant varieties, and S denotes cold-sensitive varieties.

4.12.2 *OsProT*

InDel in gene body has been used to develop DNA markers because of its practical amplification and detection. InDel, 82 bp deletions, obtained from the sequence comparison of the *OsProT* gene body from Nipponbare and 9311 (Figure 4.12.2a) were selected for marker development. The tolerant allele is 280 bp, while the sensitive allele is 198 bp. The marker showed distinct DNA patterns between tolerant and sensitive varieties on a 2 % agarose gel (Figure 4.12.2b). To validate this marker, the 159 rice varieties were genotyped. The results of genotyping showed that 63.83 % (30 out of 47) of tolerant varieties had the tolerant allele of this gene, while 73.21 % (82 out of 112) of sensitive varieties had the sensitive allele (Figure 4.12.2c). The tolerant allele was found in all tested groups (*japonica*, *indica*, admixture, and n/a). The tolerant allele was present in all tolerant *japonica* (100 %, 19 out of 19), but 23.81 % (5 out of 21) of tolerant *indica* (Figure 4.12.2d). ANOVA and regression analysis revealed a significant association ($P < 0.05$) between the allele identified by the *OsProT* marker and the cold tolerance score of 159 rice germplasm accessions under cold stress at the seedling stage. This regression analysis has an R-squared value of 0.139, indicating that the *OsProT* marker allele can explain 13.9 % of the variation in cold tolerance at the seedling stage in 159 rice germplasm, with a 95 % confidence level.

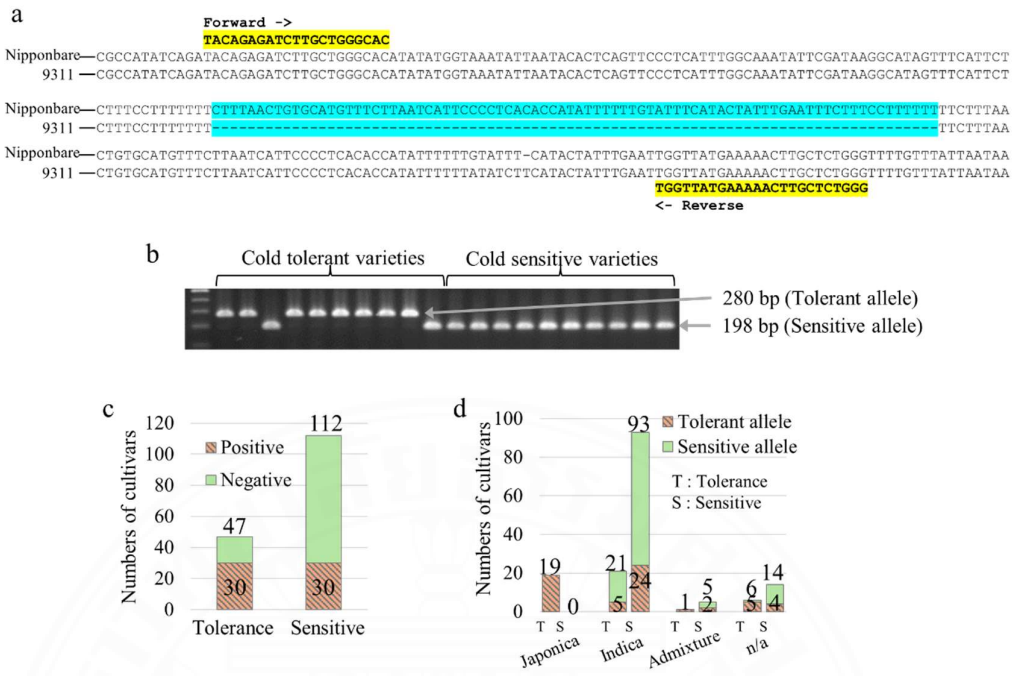


Figure 4.12.2 Development and validation of the *OsProT* InDel marker. **a**) Primers and InDel location within the OsProT gene showed an 82-bp deletion (blue stripe) observed between the cold-tolerant Nipponbare and cold-sensitive variety 9311. **b**) Representative genotyping samples include ten cold-sensitive and ten cold-tolerant varieties. Agarose gel exhibits distinct DNA bands of 280 bp and 198 bp, corresponding to the tolerant and sensitive alleles, respectively. **c**) Allele frequency of *OsProT* in extremely tolerant (47) and extremely sensitive (112) rice varieties. Orange indicates the proportion of tolerant alleles, while the green bars indicate the proportion of sensitive alleles in two rice variety groups. **d**) The distribution of tolerant and sensitive alleles of *OsProT* within each rice subspecies group is Japonica (19), Indica (114), Admixture (6), and not available (20). Here, T denotes cold-tolerant varieties, and S denotes cold-sensitive varieties.

CHAPTER 5
DISCUSSION

5.1 Effects of cold stress on rice seedling

Rice seedlings, particularly *indica* rice, face cold stress, a significant threat to rice growth and productivity (Dasgupta et al., 2020; Dong et al., 2019; Yang et al., 2015). Our study reveals a cold effect on seedlings, the cold-sensitive varieties (SPR90 and RD31) exhibit rapid leaf rolling and withering within 24 hours, while the cold-tolerant varieties (NB, B30, and DPY) insistently maintain normal morphology. This is the timing at which cold stress exposure influences phenotypic differences between tolerant and sensitive varieties. This finding is consistent with leaf rolling and withering are common markers under cold stress at the seedling stage and also reflect water deficits under severe stress (Dong et al., 2019). Our findings show that cold-tolerant seedlings (NB and B30 DPY) have high survival rates, whereas cold-sensitive seedlings (RD31 and SPR90) have low survival rates, which is consistent with previous studies that documented the survival rates of these seedling varieties under cold stress for 72 hours. (Yongbin et al., 2023).

5.2 Response mechanism of cold tolerant variety under cold stress

RNA sequencing, a powerful tool for identifying transcriptomic responses, was utilized to elucidate the mechanisms and explore novel genes associated with cold tolerance in rice seedlings. This study specifically aimed to identify genes contributing to cold tolerance at the seedling stage. We compared the transcriptomes of cold-tolerant (B30) and cold-sensitive (RD31, SPR90) varieties to unearth DEGs specific to the cold-tolerant B30. By focusing on B30's unique DEGs, we sought to elucidate the underlying mechanisms of its cold tolerance. Our analysis revealed 4046, 6139, and 4430 DEGs in B30, RD31, and SPR90, respectively, under cold stress. This observation suggests that cold-sensitive rice varieties exhibit a higher number of genetic alterations in response to cold stress compared to cold-tolerant varieties. This phenomenon might be attributed

to the cold-tolerant variety's ability to maintain stability, potentially leading to greater cold tolerance. Notably, these findings align with previous observations by Zhang et al. (2012), where the cold-sensitive variety C418 exhibited a higher number of cold-responsive DEGs compared to the cold-tolerant K354 in rice seedlings.

Among the 748 differentially expressed genes (DEGs) specifically expressed in cold-tolerant B30, transcription factors (TFs) emerged as the most enriched category, with 39 members. Notably, these TFs displayed an interesting expression pattern, predominantly upregulating genes during cold stress. These TFs belong to diverse families, including *ZFP* (9 genes), *bHLH* (7 genes), *bZIP* (7 genes), *WRKY* (4 genes), *NAC* (4 genes), *AP2/EREBP* (6 genes), and *MYB* (2 genes). This unique response pattern highlights the activation of multiple TFs in B30 during the cold stress stage (24 hours), potentially constituting cold tolerance mechanisms. Interestingly, families like *bZIP*, *bHLH*, *WRKY*, *NAC*, and *AP2/EREBP* predominantly upregulate their members, while *ZFP* shows equal up/down regulation and *MYB* displays solely downregulation. As Xie et al. (2022) described, TFs are molecular proteins that bind to DNA sequences, regulating gene expression. Extensive research has explored the critical role of TFs and their associated regulatory genes in rice's response to stress, including cold (Guan et al., 2019; Guo et al., 2019). This study aligns with previous reports suggesting the crucial role of TFs in enhancing cold tolerance (Dasgupta et al., 2020; Guan et al., 2019; Guo et al., 2019).

bZIP TFs are multifaceted regulators in plants, influencing diverse processes like stress signaling, pathogen defense, photomorphogenesis, and seed maturation (Ji et al., 2009). Stress-responsive gene expression can be regulated through both ABA-dependent and independent pathways (Dasgupta et al., 2020). In the context of abiotic stress, *bZIPs* play a key role in stress signaling via ABA-dependent pathways, where various stresses induce their expression, ultimately contributing to stress tolerance (Hossain et al., 2010; Guan et al., 2019; Xiang et al., 2008). Interestingly, this study identified several *bZIP* TFs, including *bZIP01*, *bZIP23*, *bZIP12*, and *bZIP60*, as significantly upregulated in the cold-tolerant B30 variety under cold stress. Among these, *OsbZIP23* is known for its crucial role in conferring ABA sensitivity, with its overexpression leading to enhanced drought and salt tolerance in rice (Xiang et al., 2008). Notably, the observed cold-induced upregulation of bZIPs aligns with previous

reports (Hossain et al., 2010), further highlighting their importance in this context. Additionally, *OsbZIP01*, specifically, has been implicated in the salicylic acid-dependent signaling transduction pathway and defense responses against pathogens in rice (Meng et al., 2005).

NAC TFs are known regulators of stress-responsive gene expression via ABA-dependent pathways, contributing to abiotic stress tolerance in plants (Dasgupta et al., 2020). Notably, this study identified unique upregulation of specific *NAC* TFs (*NAC2*, *ONAC045*, and *NAC122*) in the cold-tolerant B30 variety under cold stress. This finding aligns with previous reports demonstrating cold-induced upregulation of *NAC* genes and their crucial roles in rice cold tolerance (Guan et al., 2019; Zheng et al., 2009). Moreover, functional studies have shown that overexpressing *OsNAC45* enhances rice tolerance to salt and drought (Zhang et al., 2020), while *OsNAC2* regulates ABA-dependent genes and contributes to abiotic stress tolerance (Yu et al., 2021). Interestingly, *OsNAC122*, known for its role in rice disease resistance (Sun et al., 2013), suggests potential involvement in broader stress response mechanisms beyond cold tolerance.

APETALA 2/ethylene response element binding protein (*AP2/EREBP*) TFs are key regulators of stress-responsive genes, thereby enhancing plant tolerance to diverse stresses like cold and drought (Dasgupta et al., 2020; Dietz et al., 2010; Xie et al., 2022). Beyond stress responses, they also play vital roles in plant development and defense (Xie et al., 2022). Notably, several members of this TF family have been implicated in plant stress signaling pathways (Guan et al., 2019), and their suppression leads to disruptions in various developmental processes (Dietz et al., 2010; Xie et al., 2022). Moreover, overexpressing *AP2/EREBPs* in *Arabidopsis thaliana* has been shown to enhance salt and cold tolerance (Xie et al., 2022). In rice, research shows that ABA, ACC, salt, and cold rapidly induce *OsLG3*, whose overexpression improves salt and osmotic tolerance (Xiong et al., 2018; Wang et al., 2020). Interestingly, this study identified significant upregulation of *OsERF#007* and *OsLG3*, both encoding AP2 domain-containing proteins, specifically in the cold-tolerant B30 variety under cold stress.

Under cold stress, specific *WRKY* TFs (*WRKY46*, *WRKY60*, and *WRKY15*) were significantly upregulated only in the cold-tolerant B30 variety. This finding aligns

with established roles of *WRKYs* in abiotic stress responses, where they regulate stress-responsive genes through both ABA-dependent and independent pathways (Dasgupta et al., 2020; Guan et al., 2019). Notably, *WRKY* genes display differential expression patterns under cold stress, with high expression of specific members like *WRKY70* contributing to plant cold tolerance (Guo et al., 2019). Beyond cold stress, WRKYs are extensively studied for their involvement in both rice plant-pathogen responses and cold stress adaptation (Dasgupta et al., 2020; Pan et al., 2020). Furthermore, *WRKYs* play crucial roles in regulating various other plant pathways (Guo et al., 2019).

Under cold stress, the B30 variety exhibited significant upregulation of several *bHLH* TFs, including *OsbHLH150*, *OsbHLH151*, *OsbHLH061*, *OsbHLH138*, and *Os01g0626900*. In general, bHLH proteins, characterized by their basic helix-loop-helix domain, function as key regulators of diverse physiological processes and biosynthetic pathways through their control of target gene expression (Hao et al., 2021). Notably, *OsbHLH138* plays a specific role in thermosensitive male sterility by interacting with and activating the *TMS5-2* promoter, influencing rice fertility (Wen et al., 2019). Beyond their specific functions, bHLH proteins have well-established roles in abiotic stress responses, including cold stress, where cold stress can induce these TFs (Guo et al., 2019; Yan et al., 2023). For instance, a bHLH protein in *Arabidopsis* directly binds to the *CBF3* promoter, promoting its expression and ultimately enhancing cold tolerance (Chinnusamy et al., 2003). This highlights the potential importance of the *bHLH* family in stress responses, one of the largest TF families in *Arabidopsis* (Hao et al., 2021).

GO and KEGG enrichment analyses revealed insights into DEGs within the cold-tolerant B30 variety. Unique GO enrichments in the cold-tolerant B30 variety highlight metal ion transport, plastid/chloroplast functions, and organelle membrane compositions, suggesting potential mechanisms underlying its enhanced tolerance. This corresponds with the Do Amaral et al. (2016) study, which found that chloroplasts and plastids are highly sensitive to abiotic stress, including cold, in rice seedlings. Furthermore, membranes act as critical barriers between organelles and their surroundings, as well as being sensitive to temperature changes. Cold stress significantly disrupts the function of various organelle membranes, including photosynthetic membranes, chloroplast membranes, thylakoid membranes, and other

biomembranes, in rice seedlings (Liu et al., 2022). This suggests that B30 may have altered organelle structures or functionalities, which may contribute to enhanced membrane stability and fluidity for improved environmental adaptation (Liu et al., 2022). Moreover, B30 exhibited enrichments in metabolic pathways, secondary metabolite biosynthesis, and nitrogen metabolism. This supports previous findings about the roles of metabolic pathways and secondary metabolite biosynthesis in stress response and adaptation (Chen et al., 2022; Jeyasri et al., 2023; Reshi et al., 2023). Cold stress typically disrupts overall metabolism and nitrogen metabolism (Do Amaral et al., 2016), while plant secondary metabolites are crucial for resilience (Chen et al., 2022; Jeyasri et al., 2023; Reshi et al., 2023). The studies also show that cold stress disrupts and decreases nitrogen metabolism in rice plants by downregulating nitrogen metabolism-related genes. Consistent with this, Thapa et al. (2023) observed alterations in nitrogen metabolism-related DEGs under cold stress, further supporting our findings.

5.3 Cold tolerant genes

Cold stress stimulates distinct expression patterns for *OsFH10, ONAC045, SnRK2, OsISA1, OsFER1*, and *OsProT* genes, differentiating cold-tolerant from cold-sensitive rice varieties. Notably, these unique expression profiles suggest their specific roles in the cold stress response of the cold-tolerant varieties. To gain insight into their potential regulatory mechanisms, we selected these six DEGs for further investigation.

5.4 *OsFH10*

Cold stress significantly upregulates *OsFH10* expression in tolerant rice varieties, which might implicate its involvement in cold stress tolerance. This aligns with reports of *OsFH10* induction by drought and cadmium stress (Li et al., 2023), implying a broader role in abiotic stress responses. *OsFH10* encodes a formin-like protein 10, belonging to a family of actin-binding proteins (ABPs) crucial for fundamental cytoskeleton processes like construction, polarization, and division. Several formins influence actin remodeling, modulate cell wall morphology, and control plant growth and development (Li et al., 2023; Ren et al., 2022; van Gisbergen &

Bezanilla, 2013). As crucial regulators of actin dynamics in plants, formin proteins, through their FH2 domain, mediate the interaction of actin and microtubule cytoskeletons (Cvrčková et al., 2015; Li et al., 2023). Plant cytoskeletons, composed of actin filaments and microtubules, regulate plant growth, development, and stress responses by reshaping cells for environmental adaptation (Li et al., 2023; Wang et al., 2022). Notably, the actin cytoskeleton regulates plant response and tolerance to diverse stresses (Kumar et al., 2023; Wang & Mao, 2019; Wang et al., 2021; Wang et al., 2022). Previous research has shown that microtubule dynamics play a role in plant cold tolerance via a polymerization mechanism and in maintaining cell stability such as morphology, movement, and transport (Ma & Liu, 2019). Furthermore, overexpressing the *actin-depolymerizing factor 3 (ADF3)* gene in rice enhanced cold tolerance (Byun et al., 2021). Therefore, elevated *OsFH10* expression in cold-tolerant rice suggests its likely contribution to cold tolerance through cytoskeleton dynamics, potentially via altered morphology and enhanced cell stability. However, our understanding of the functions of the *OsFH10* gene, particularly in response to environmental stresses in rice, remains limited (Li et al., 2023).

Environmental stresses like cold, salt, drought, and heavy metals can induce DNA methylation (Dubin et al., 2015; Ferrari et al., 2020; Ferreira et al., 2015; Gonzalez et al., 2013; Wang et al., 2011), which is crucial for regulating gene expression and the plant's abiotic stress response (Lim et al., 2019; Pan et al., 2011; Yaish, 2013). This study offers into the potential role of DNA methylation in cold tolerance through the *OsFH10* gene. Our findings reveal a striking difference in DNA methylation patterns between cold-tolerant and sensitive rice varieties. In cold-sensitive varieties, the *OsFH10* promoter region exhibits complete methylation (100 %) under both cold and control conditions, whereas in cold-tolerant varieties, it remains unmethylated (0 %). This suggests that DNA methylation of the *OsFH10* promoter might contribute to cold sensitivity by silencing gene expression, supporting the theory that DNA methylation in the promoter CpG island inhibits transcription (Guo et al., 2019; Lim et al., 2019). Notably, although cold stress itself doesn't induce methylation in this region, this unique pattern appears specific to cold-sensitive varieties, offering a potential avenue for further investigation. Meanwhile, DNA methylation patterns of the gene body are similar in all cold-tolerant and sensitive varieties, with partial methylation (1–99 %) observed under

both conditions. This suggests that DNA methylation in the *OsFH10* gene body does not regulate alternative splicing under cold stress.

Plant cold stress responses often involve intricate regulatory mechanisms, including intron2 retention (IR2) events. IR plays a crucial role in plant abiotic stress tolerance (Dinh et al., 2016; Gu et al., 2014; Lee et al., 2016). IR, the most common form of AS, plays a pivotal role in regulating gene expression, particularly in stress-tolerant genes (Ganie & Reddy, 2021). To explore the effect of cold stress on AS of *OsFH10*, it was investigated using semi-RT PCR. Our findings revealed that cold stress induces IR in *OsFH10*, specifically in cold-tolerant rice varieties. This suggests contrasting IR mechanisms for adaptation between tolerant and sensitive rice varieties to cold stress. Furthermore, cold stress induces IR to appear specific to target genes and varieties. This supports previous observations, highlighting the dynamic of cold-induced IR, which varies based on gene targets, organelles, and plant cultivars (Lee et al., 2016; Ganie & Reddy, 2021). Consistent reports have shown cold-induced IR events in other cold-responsive genes in rice seedlings (Lee et al., 2016; Dinh et al., 2016). Considering the paucity of information on *OsFH10*'s AS responses to abiotic stress, our findings provide compelling evidence for the potential contribution of *OsFH10*'s IR to cold tolerance in rice seedlings.

5.5 *OsISA1*

OsISA1, encoding isoamylase 1, is a starch-debranching enzyme crucial for amylopectin synthesis, impacting rice endosperm development, seed development, and germination (Kubo et al., 1999; Utsumi et al., 2011; Du et al., 2018). This enzyme promotes the formation of insoluble glucans and crystallization-competent amylopectin (Smith et al., 2020; Boehlein et al., 2023). *OsISA1* exhibits dramatic upregulation in cold-tolerant rice varieties under cold stress, contrasted by negligible upregulation in cold-sensitive cultivars. Similarly, high temperatures induce *OsISA1* upregulation during the milky stage of Nipponbare rice grains (Yamakawa et al., 2007). Yeast studies further support this, revealing a significant increase in semi-crystalline α-glucan abundance with the presence of maize *ISA1* compared to its absence (Boehlein et al., 2023). Interestingly, *OsISA1* belongs to the starch and sucrose metabolism pathway,

known to influence cold tolerance in rice seedlings (Yan et al., 2023). Though cold reduces photosynthesis and subsequently starch synthesis (Smith et al., 2020), cold-tolerant rice retains these activities via upregulating *ISA1*, potentially contributing to their stress tolerance. Despite existing research on *OsISA1*'s role in rice development, reports on its cold stress response are very limited. Despite extensive research on *OsISA1* in development, its role in stress responses remains largely unexplored (Thalmann et al., 2016). This study sheds light on its differential expression under cold stress, but further investigation is needed to elucidate its precise role in plant stress tolerance.

This study explored the potential association between alternative splicing (AS) of the *OsISA1* gene, specifically intron retention (IR), and cold tolerance in rice seedlings. While extensive research exists on *OsISA1*'s functions, its involvement in AS responses to cold stress remains largely unexplored. This study investigated the effect of cold stress on the AS event of *OsISA1*, revealing two transcripts, normal and IR. Notably, all cold-tolerant varieties exhibited upregulation of this IR isoform under cold stress. This suggests that the presence of IR in *OsISA1* mRNA might contribute to cold stress adaptation. These findings align with Teng et al., (2022), who reported AS variations in *OsISA1* during soil drying in rice spikelets. Collectively, our results provide valuable insights into the molecular mechanisms underlying the contribution of the *OsISA1* gene to cold tolerance in rice seedlings.

5.6 *ONAC045*

ONAC045 encodes NAC domain-containing protein 45, a member of the plant-specific *NAC* transcription factor family that plays a known role in stress responses and development (Diao et al., 2020; Zhang et al., 2020; Zheng et al., 2009). Several *NAC* TFs enhance plant cold tolerance (Diao et al., 2020). Our study revealed that *ONAC045* expression is specifically upregulated in cold-tolerant rice varieties under cold stress, suggesting its potential role in this response. This finding aligns with reports of improved stress tolerance upon *GmNAC20* overexpression in rice (Yarra and Wei, 2021). Moreover, *ONAC045* overexpression enhances drought and salt tolerance

(Zheng et al., 2009), and mutants exhibit increased salt stress sensitivity (Zhang et al., 2020).

Interestingly, cold stress induced upregulation of AS isoforms, IR2 and IR1+IR2, of *ONAC045* exclusively in cold-tolerant varieties. This suggests a potential link between AS and enhanced cold tolerance in rice seedlings. The results indicate that cold stress induces IR specific to target regions and varieties. These findings align with previous reports of cold-induced IR isoforms in cold-responsive genes in rice seedlings (Dinh et al., 2016; Lee et al., 2016). Previous studies have mainly focused on drought and salt stress, while cold stress has few reports (Diao et al., 2020). Interestingly, these stresses share similar responses in plants, like water balance and hormone signaling (Ramkumar et al., 2022; Sharma et al., 2013). This overlap suggests potential for *ONAC045*, a known drought/salt tolerance factor (Zheng et al., 2009), to also play a role in cold tolerance. Our study highlights a potential role for *ONAC045* in rice cold tolerance, expanding its broader role in abiotic stress responses, particularly cold responses. The observed AS response under cold stress suggests a novel regulatory mechanism for *ONAC045*.

Promoter polymorphisms can influence gene expression (Wang et al., 2021b). Our analysis of the *ONAC045* promoter revealed two SNPs near the TATA-box region. TATA-boxes are a critical element for regulating transcription (Savinkova et al., 2023). Since TFs interact with the TATA-box and other cis-elements to initiate transcription (Kim et al., 2020), these promoter SNPs could potentially alter TF binding, impacting expression efficiency and ultimately protein production (Morgil et al., 2020). This aligns with the observed differential expression of *ONAC045*, with higher levels in cold-tolerant varieties compared to cold-sensitive ones. This finding aligns with reports demonstrating how promoter SNPs can influence promoter activity, thereby regulating gene expression and protein production (Morgil et al., 2020). Furthermore, *NAC* gene promoters often contain cis-acting elements responsive to abiotic stresses like drought and salinity (Tran et al., 2004), supporting a potential role for *ONAC045* in cold stress response. Since transcriptional processes can influence AS events (Kadener et al., 2002; Kolathur, 2021), the identified promoter polymorphism might modulate AS in *ONAC045*, leading to the generation of diverse IR transcripts under cold stress. This finding aligns with prior reports demonstrating cold-induced

upregulation of IR isoforms in cold-responsive genes in rice seedlings (Dinh et al., 2016; Lee et al., 2016). Further investigation is needed to elucidate the specific role of *ONAC045* in the cold tolerance of rice seedlings.

5.7 *SnRK2*

SnRK2, a member of the *Snf2* protein family, functions as a chromatin remodeler (CHR) by altering nucleosome positioning in chromatin (Guo et al., 2022; Hu et al., 2013; Song et al., 2021). CHRs are crucial regulatory mechanisms for stress-responsive gene expression in plants (Song et al., 2021). The diverse *Snf2* family in rice plays vital roles in development and adaptation to abiotic stresses like salt, drought, cold, and ABA signaling (Hu et al., 2013; Guo et al., 2022; Kulik et al., 2011). Our study revealed that *SnRK2* expression was high only in specific cold-tolerant varieties under both control and cold stress conditions. Interestingly, only these varieties displayed upregulation of diverse AS isoforms and skipped exon forms under cold stress, leading to transcript diversity. This suggests that cold stress induces upregulation of *SnRK2* transcript isoforms through AS, potentially resulting in functionally distinct protein products encoded by different exon combinations. Notably, these alternative expression patterns are exclusive to cold-tolerant rice seedlings. Supporting these findings, Hu et al. (2013) demonstrated that many *Snf2* family genes are essential for plant growth and stress response, with their expression and function responding to drought, salt, and cold stresses. Additionally, Yang et al. (2019) showed that mutations in the *Arabidopsis PKL* gene, a member of the *Snf2* family, increase cold stress sensitivity, supporting the significance of *SnRK2* in rice cold tolerance.

Analysis of *SnRK2* promoter sequences revealed an interesting SNP located near the TSS that is different between cold-tolerant and cold-sensitive rice varieties. This SNP may contribute to the observed differences in gene expression patterns between these varieties. SNP variations within promoters are known to influence gene expression by affecting the binding of TFs (Morgil et al., 2020). The SNP identified near the *SnRK2* TSS might influence gene expression patterns by altering the binding of TFs. This could potentially explain the observed cold stress-induced generation of various skipped exon transcripts within *SnRK2*. This finding aligns with previous

reports demonstrating the influence of promoter variations on AS (Kolathur, 2021; Xin et al., 2008). AS is regulated by a complex network of splicing factors. Among these, trans-acting splicing factors play a particularly crucial role (Deepika et al., 2024). Promoters, located upstream of the TSS, function as cis-acting elements. They interact with trans-acting factors to regulate gene transcription (Kadener et al., 2002; Kolathur, 2021). In the context of *SnRK2*, the promoter SNP might influence the binding of trans-acting splicing factors, leading to the generation of diverse transcripts through AS under cold stress conditions.

However, there have been very few reports on the function of cold tolerance of *SnRK2* in rice. This study demonstrates that various expression patterns of *SnRK2* may potentially link enhanced cold stress tolerance to rice seedlings. Further investigation is required to elucidate the specific roles of each isoform and their potential contribution to cold stress response mechanisms in rice.

5.8 *OsFER1*

Under cold stress, expression of *OsFER1* was high in cold-tolerant varieties, indicating that these genes may be related to cold-tolerance seedlings. *OsFER1* encodes the ferritin protein, which stores iron in cells and protects it from reactions with other molecules (Gross et al., 2003; Paul et al., 2016; Nguyen et al., 2022). Ferritin also assists in the defense against Fe toxicity by transferring Fe^{2+} (the reactive form) to Fe^{3+} (the non-reactive form) during stress responses (Nguyen et al., 2022). Furthermore, ferritin gene expression in plants can improve abiotic stress tolerance associated with ROS scavenging, such as *TaFER-5B* in Arabidopsis, which improved heat stress tolerance (Zang et al., 2017). *OsFER1* was found to be upregulated in the cold-tolerant B30 variety in this study, suggesting that the expression of this gene could be related to rice seedling cold tolerance. Since the ferritin protein regulates the equilibrium of iron uptake and oxidative stress in plants, this influences abiotic stress tolerance (Nguyen et al., 2022). Although the *OsFER1* protein is essential for a mechanism that enables plants to adapt to environmental stress, little is information about the function of this gene. This study demonstrates that expression at a high level of the *OsFER1* may potentially exhibit enhanced cold stress tolerance in the rice

seedling stage. Indicates that expression has a positive association with cold tolerance via the upregulation of these stress-responsive genes. These genes may respond to several abiotic stresses since they are members of gene families that are important for various stress responses.

Cold stress exposure resulted in the upregulation of *OsFER1* expression exclusively in cold-tolerant rice varieties. Notably, only these varieties displayed upregulated transcript abundance of three distinct IR isoforms: IR2, IR3, and IR2+IR3. These findings suggest a potential link between *OsFER1* expression patterns and cold tolerance in rice seedlings. Indicates that IR isoforms has a positive association with cold tolerance via the upregulation of these stress-responsive genes. These genes may respond to several abiotic stresses since they are members of gene families that are important for various stress responses.

5.9 *OsProT*

OsProT, encoding proline transporter 1, localizes in the plasma membrane and facilitates proline uptake, enabling proline accumulation within cells. Proline acts as an osmolyte, enhancing plant tolerance to various stresses like drought, salt, nutrient deficiency, and heat by accumulating during stress conditions (Lin et al., 2019). This accumulation, mediated by *OsProT* expression, enhances plant tolerance to these stresses (Sun et al., 2010). Our study revealed upregulated *OsProT* expression specifically in cold-tolerant rice varieties under cold stress, suggesting a potential role in cold tolerance in rice seedlings. Furthermore, cold stress induced upregulation of an IR isoform (IR4+IR7) of *OsProT* exclusively in cold-tolerant varieties. This finding is consistent with previous reports of cold-induced IR isoforms in cold-responsive genes of rice seedlings (Dinh et al., 2016; Lee et al., 2016). Prior to this research, limited information existed regarding *OsProT*'s AS response to cold stress. These findings contribute to the understanding of *OsProT* expression and its specific AS response under cold stress might contribute to enhanced cold tolerance in rice seedlings. Since these genes belong to gene families involved in various stress responses, they may contribute to tolerance against multiple abiotic stresses.

Variations within promoter sequences influence gene expression through regulatory elements that interact with TFs (Huluka & Kumsa, 2022; Villao-Uzho et al., 2023). Our analysis of the *OsProT* promoter in cold-tolerant and cold-sensitive rice varieties revealed significant polymorphisms, including SNPs and InDels near TSS. These polymorphisms are located in the DRE core and STRE element. Cis-regulatory elements within promoters play a pivotal role in gene regulation by interacting with specific TFs (Huluka & Kumsa, 2022). Among these, the STRE element acts as a crucial activation site for heat shock factors (HSFs) under various stress conditions, particularly heat shock in eukaryotes (Guo et al., 2008; Haralampidis et al., 2002). In *Arabidopsis*, upon heat stress, *HsfA1* TFs bind to the STRE element in *HSP* genes, inducing expression and enhancing thermotolerance (Guo et al., 2008). Furthermore, STRE deletion in the *AtHsp90-1* gene promoter reduced gene expression under heat stress, highlighting its critical role in regulating promoter activity (Haralampidis et al., 2002). The presence of short deletions near the TSS suggests a DRE core motif. Similar to the DRE, this motif interacts with DREB TFs, regulating gene expression (Dubouzet et al., 2003; Narusaka et al., 2003; Sakuma et al., 2006). In both *Arabidopsis* and rice, DRE motifs within stress-responsive gene promoters facilitate activation under drought, salt, and cold stress conditions by directly interacting with DREB proteins, ultimately enhancing tolerance (Dubouzet et al., 2003; Sakuma et al., 2006).

Our findings revealed SNPs and InDels near the TSS region, residing within regulatory elements. These polymorphisms potentially contribute to the observed differential gene expression patterns between cold-tolerant and cold-sensitive rice varieties, potentially influencing cold tolerance adaptation in seedlings. This is consistent with the established role of cis-regulatory elements interacting with TFs to regulate gene expression during responses to environmental tension in plants (Brooks et al., 2023). This suggests that IR isoform expression of *OsProT*, observed only in cold-tolerant varieties under cold stress, might be controlled by these polymorphisms. This finding is further supported by evidence that minimal promoter changes can significantly alter splicing patterns (Cramer et al., 1997). Additionally, IR is known to regulate key genes in gene expression (Wang et al., 2006). Consequently, *OsProT* emerges as a promising candidate for marker development aimed at enhancing cold tolerance in rice seedling breeding.

5.10 *ONAC045* and *OsProT* Markers

The SNP within the *ONAC045* promoter region was exploited to develop a DNA marker for cold tolerance in rice using the tetra-primer PCR method. The PCR products exhibit a clear size difference (39 bp) between cold-tolerant and cold-sensitive alleles, readily detectable on general agarose gels, making it suitable for general laboratory use. Both alleles can be amplified in a single PCR reaction, reducing costs and analysis time. This suggests that the *ONAC045* marker has several advantages, including ease of use and cost-effectiveness. Similar to previous studies, SNP markers were developed from SNPs in the promoters of Gn1a and GS5 using the tetra-primer PCR method, and these markers are reliable (Kim et al. 2016). The *ONAC045* marker displayed a significant association ($P < 0.01$) with cold tolerance scores in validation using 159 rice germplasm lines. Cold-tolerant varieties possessed a significantly higher frequency (51.06 %) of the tolerant allele compared to cold-sensitive varieties (0.89 %). This association suggests a potential link between the marker genotype and cold tolerance phenotype. The *ONAC045* marker demonstrated high efficacy in predicting cold tolerance within *japonica* rice varieties. It accounted for an accuracy of approximately 43.8 % in predicting tolerance in germplasm rice seedlings. However, sensitivity within *indica* varieties was limited due to the low frequency of the tolerant allele in cold-tolerant *indica*. These findings suggest that the *ONAC045* marker is a valuable tool for identifying cold-tolerant *japonica* rice cultivars with high accuracy. Further research is needed to develop complementary markers for *indica* rice varieties.

In plant breeding, insertions/deletions (InDels) exceeding 10 bp are valuable genetic markers due to their prevalence in plant genomes and ease of detection (Li et al., 2023; Long et al., 2022). The rise of next-generation sequencing technologies has further fueled the development and application of InDel markers in marker-assisted breeding strategies (Hu et al., 2020; Li et al., 2023; Long et al., 2022). This study exploited an InDel polymorphism within the *OsProT* gene to develop a DNA marker for cold tolerance in rice. This marker offers practical advantages. PCR products exhibit a clear size difference (82 bp) between cold-tolerant and cold-sensitive alleles, readily detectable on general agarose gels. Validation in 159 rice germplasm lines revealed a significant association ($P < 0.01$), indicating a potential link between the *OsProT* marker

genotype and cold tolerance. Cold-tolerant varieties possessed a significantly higher frequency (63.83 %) of the tolerant allele compared to cold-sensitive varieties. Notably, the tolerance allele was present in all tolerant *japonica* accessions (100 %) but only 23.81 % of tolerant *indica* accessions. Furthermore, the *OsProT* marker had more sensitivity to cold-tolerant *indica* seedlings than the *ONAC045* marker, despite being less accurate. This indicates that the *OsProT* marker is an effective tool for identifying cold-tolerant *indica* rice cultivars. The validation of the *OsProT* marker contributes to the growing body of evidence supporting the use of InDel markers in rice breeding programs, as demonstrated in previous studies (Hu et al., 2020; Whankaew et al., 2020; Yongbin et al., 2023).

This study introduces two novel markers for cold tolerance in seedlings: the *ONAC045* SNP marker and the *OsProT* InDel marker. These markers offer significant advantages due to both markers are detectable using standard agarose gel electrophoresis, a common and cost-effective technique readily available in general laboratories. The *OsProT* InDel marker shows particular potential for cold-tolerant *indica* rice breeding, as it is more sensitive in identifying tolerant alleles than the *ONAC045* SNP marker, which is more accurate for cold-tolerant *japonica* rice. These markers facilitate the identification of germplasm carrying novel cold-tolerance genes, a critical step for developing new cold-tolerant varieties. The development of these markers aligns with the importance of focusing on discovering novel sources of cold tolerance for future breeding programs (Dar et al., 2021; Kanngan et al., 2023).

CHAPTER 6
CONCLUSIONS

This study aimed to identify genes unique to the cold-tolerant rice variety at the seedling stage, using RNA sequencing. This approach led to the discovery of novel genes, 748 DEGs specific to B30, suggesting their role in cold tolerance. This study reports the identification of genes related to cold tolerance, including *OsFH10*, *ONAC045*, *SnRK2*, *OsISA1*, *OsFER1*, and *OsProT*. The expression of these genes has a positive association with cold tolerance via upregulation. To further understand the gene expression associated with cold tolerance in rice seedlings, this study investigated the expression patterns, DNA methylation, AS events, and promoter polymorphisms of cold-responsive genes. This study suggests a potential link between the AS events in all genes of interest and the enhanced cold tolerance phenotype, which might play a role in facilitating improved cold adaptation in rice seedlings. Additionally, variations in the core promoters of *SnRK2*, *ONAC045*, and *OsProT* might influence gene expression patterns and potentially contribute to the differences in cold tolerance between varieties. This research has led to the development of *ONAC045* SNP and *OsProT* InDel markers associated with cold tolerance. These markers offer a simple and effective tool for breeding cold-tolerant rice varieties, particularly InDel *OsProT* markers, which enhance the accuracy of selection for cold-tolerant *indica* rice. Overall, the study provides insights into the cold response mechanisms of these genes and their potential use in developing cold-tolerant rice varieties. This knowledge can also be applied to understanding other abiotic stresses in rice. However, further research is needed to definitively confirm the specific roles of *OsFH10*, *ONAC045*, *SnRK2*, *OsISA1*, *OsFER1*, and *OsProT* in rice seedling cold tolerance.

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