Unravelling the role of Jasmonic acid during cold tolerance in rice (*Oryza sativa* L.)

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Karlsruhe, im December 2020

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ABBREVIATIONS

12OPDA: 12-oxo-phytodienoic acid

13-HODE: 13-hydroperoxy-octadecatrienoic acid

13-LOX: 13- lipoxygenase

$^1O_2$: Singlet oxygen

ABA: Abscisic acid

ABF: ABRE binding transcription factor

ABRE: ABA-responsive element

AOC: Allene oxide cyclase

AOS: Allene oxide synthase

AP2/EREBP: APETELA2/ethylene-responsive element-binding protein

AP2/ERF: APETELA2/ethylene responsive factor

bZIP: Basic leucine zipper transcription factor

CBF: C-repeat binding factor

COI1: CORONATINE INSENSITIVE 1

COR: Cold responsive elements

COR: Cold-responsive protein

***cpm2*:** Coleoptile photomorphogenesis 2

CRT: C repeat responsive element

CTS: COMATOSE

DMSO: Dimethyl sulfoxide

DRE: Dehydration-responsive element

DREB: Dehydration response element binding

H_2O_2: Hydrogen peroxide

IAA: Indole-acetic acid

ICE1: Inducer of CBF expression

JA: Jasmonic acid

JA-Ile: Jasmonoyl-isoleucine

JAZ: JASMONATE ZIM-DOMAIN

MAPK: MAP kinase

MAPKK: MAP kinase kinase

MDA: Malondialdehyde

MeJA: Methyl jasmoante

NACRS: NAC recognition sequence.

NOG1-2: Nucleolar GTP-Binding Protein 1-2

O^-_2: Superoxide radical

OH: Hydroxyl radical

OPC-8:0: 3-oxo-2-(cis-2-pentenyl)-cyclopentane-1-octanoic acid

OPR: OPDA-Reductase

OsNAC: NAM-ATAF1,2-CUC2

PCD: Programme cell death

PLD: Phospholipase D

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RWC: Relative water content

SA: Salicylic acid

SA: Salicylic acid

SCF: SKP1, Cullin, F-Box-Protein

TFs: Transcription factors

UPLC-MS: Ultra-performance liquid chromatography-tandem mass spectrometry

WT: Wild type

α-LeA: α-linolenic acid

ZUSAMMENFASSUNG

Reis ist das Grundnahrungsmittel und eine wichtige Kohlenhydratquelle für über die Hälfte der Weltbevölkerung. Kältestress ist eine an Bedeutung gewinnende abiotische Bedrohung aufgrund drastischer globaler Klimaveränderungen, die in manchen Regionen eine Kombination früher Warm- und später Kaltphasen oder ungewöhnliche Frostereignissen verursachen. Kältestress schränkt das Wachstum und die Entwicklung von Reispflanzen im Keimlingsstadium stark ein, die Keimung und das Bestockungswachstum wird gehemmt. Im Fortpflanzungsstadium werden Mechanismen der männlichen Befruchtung gestört, was den Ertrag deutlich mindert. Die Abwehrreaktion von Pflanzen auf Kältestress umfasst ein sehr komplexes Netzwerk von Signalkaskaden, an denen viele Transkriptionsfaktoren und Phytohormone beteiligt sind. Jasmonsäure (JA) ist eines der wichtigsten Pflanzenhormone, die das Wachstum und die Entwicklung der Pflanze regulieren und die Abwehrreaktion gegen abiotischen und biotischen Stress induzieren, ihre Rolle in der Reaktion auf Kältestress ist in Reis jedoch noch nicht gut untersucht.

In unserer Studie verwendeten wir die JA-defizienten Mutanten *cpm2* und *hebiba* sowie den Wildtyp, um die Rolle von JA bei der Reaktion von Reis auf Kältestress aufzuklären. Im ersten Teil der Studie wurden physiologische und biochemische Parameter bei JA-defizienten Mutanten und beim Wildtyp analysiert, und es wurde beobachtet, dass der Wildtyp aufgrund der geringeren Akkumulation von Malondialdehyd (MDA), des höheren Chlorophyllgehalts, des größeren Trockengewichts und des insgesamt stärkeren Keimlingswachstums unter Kältestress besser abschnitt als die JA-defizienten Mutanten. Im zweiten Teil der Studie wurde eine

Transkriptionsanalyse des kälteinduzierten Gens *OsDREB1A*, der JA-induzierten Gene *OsJAZ12* und *OsJAZ13* sowie der JA-Biosynthesegene *OsOPR7* und *OsAOS2* und Hormonmessungen durchgeführt. Die Hochregulierung des kälteinduzierten Gens *OsDREB1A* in Wurzeln und im Spross des Wildtyps, so wie in den Wurzeln der Mutanten, weisen auf eine organspezifische Kälteregulation von *OsDREB1A* hin, d.h. das Gen ist JA-abhängig im Spross und JA-unabhängig in der Wurzel. Basierend auf unseren Ergebnissen stellen wir fest, dass JA die Expression von CBF/DREB1A in einem komplexen Prozess reguliert und damit einen Kältetoleranzmechanismus im Wildtypspross induziert. Im dritten Teil der Studie klärten wir die Rolle des sekundären Botenstoff Phosphatidinsäure (PA) in der Aktivierung des stressaktivierbaren Gens *OsDREB1A* auf. Einige Publikationen deckten durch Kältestress verursachte biochemische Veränderungen auf, wie den Einstrom von Ca^{2+}, die Aktivierung sekundärer Botenstoffe wie PA, die Aktivierung von MAP-Kinasen und damit die Aktivierung stressabhängiger Gene. Wir wollten Einblick bekommen, ob solche Signalleitungselemente die Jasmonatantwort beeinflussen. Zu diesen Veränderungen zählt man die Modifikation einer Klasse von Phospholipiden, Phosphatidylcholine, die ein Bestandteil der Lipiddoppelmembran von Zellen sind. Das Lipidsignalleitungsenzym Phospholipase D (PLD) wird durch Kältestress aktiviert und hydrolisiert Phosphatidylcholin in den sekundären Botenstoff PA und Cholin. Da Kältestress zuerst an der Plasmamembran wahrgenommen wird, gehört dieser Mechanismus zu den frühen biochemischen Veränderungen. Deshalb haben wir in unserer Studie 0.5% n-butanol (v/v) appliziert, um die die Aktivität von PLD zu beeinflussen, da PLD so anstatt der Synthese des aktiven PA vermehrt die des inaktiven Phosphatidylbutanol katalysiert. Wir wollten wissen, ob die Blockade der PA-

Signalleitung in Reiswurzeln unter Kältestress die Induktion von *OsDREB1A* unterbindet, also ob die JA-unabhängige Aktivierung des Transkriptionsfaktors in Wurzeln von PA abhängig ist und ob die Antwort von JA-abhängigen Genen betroffen ist. *OsDREB1A* Transkription wurde jedoch nur teilweise in der Wurzel des Wildtyps gehemmt, was darauf hinweist das ein unbekannter Fakor ‘X’, nicht JA oder PA, zur Induktion von OsDREB1A unter Kältestress beiträgt. Die transkriptionelle Expression von *OsAOS2*, *OsJAZ12* und *OsJAZ13* wurde durch die Applikation von n-butanol hingegen vollständig gehemmt, was auf eine starke Beeinflussung des JA-Signalwegs durch PA hindeutet.

ABSTRACT

Rice is the staple food and important source of carbohydrate for over half of the world population. Cold stress is an emerging abiotic threat due to drastic global climate change which in some regions causes a combination of early warm phases and late cold or frost events. It severely limits the growth and development of rice plants at the seedling stage, and inhibits germination and tillering. At the reproductive stage, it disturbs male fertilization and reduces the yield drastically. In order to defend against cold stress plants have established a very complex network of signalling cascades with the involvement of many transcription factors and phytohormones. Jasmonic acid (JA) is one of the major plant hormones that regulates the growth and development of the plant as well as induces defence responses against abiotic and biotic stress, however its role in the response to cold is not well investigated in rice.

In our study, we used the JA-deficient mutants *cpm2* and *hebiba*, and wild type to elucidate the role of JA under cold stress. In the first part of the study analysis of physiological and biochemical parameters revealed that the wild type performed better than JA-deficient mutants, as wild type accumulated less malondialdehyde (MDA), more chlorophyll, more dry weight, and was characterized by an overall strong seedling growth under cold stress. In the second part of the study transcriptional analysis of the cold-responsive gene *OsDREB1A,* JA-responsive genes *OsJAZ12*, *OsJAZ13,* and JA-biosynthesis genes *OsOPR7*, *OsAOS2* and hormonal measurements has been conducted. Upregulation of *OsDREB1A* in shoot and root of wild type as well as in root of JA-deficient mutant indicates organ-specific cold regulation of *OsDREB1A* i.e. JA-dependent in shoots and JA-independent in roots. Based on our result

we propose, JA regulates the CBF/DREB1A expression in a complex process and induces cold tolerance mechanisms in the shoots of the wild type. In the third part of the study, we elucidated the role of the secondary messenger phosphatidic acid (PA) to activate stress-responsive genes *OsDREB1A*. Some reports revealed the cold stress causes biochemical changes such as influx of Ca^{2+}, activation of the secondary messenger like PA, activation of MAP kinase cascades, and thus activates stress-responsive genes, and we wanted to get insight whether those signalling events influence the jasmonate response. Amongst those changes a class of phospholipids, phosphatidylcholines, which are a component of the lipid bilayer structure of plasma membrane have been found to be involved into early cold signalling. The lipid signalling enzyme phospholipase D (PLD) is activated by cold stress and hydrolyses phosphatidylcholine into the secondary messenger PA and choline. As cold stress is first sensed by the plasma membrane it belongs to those early biochemical changes. Therefore, in our study, we used 0.5% n-butanol (v/v) to manipulate the activity of PLD so that an inactive phosphatidylbutanol was produced instead of an active secondary messenger PA. We wanted to know if the blocking of PA under cold stress in rice roots can inhibit the expression of *OsDREB1A,* or in other word if JA-independent activation of this transcription factor in roots is dependent on PA, and whether the response of JA-dependent genes was affected. However, *OsDREB1A* induction could not be blocked by n-butanol entirely, indicating that there is unknown factor 'X' other than JA and PA contributing to induce the expression of *OsDREB1A* under cold stress. In contrast, the transcriptional expression of *OsAOS2*, *OsJAZ12* and *OsJAZ13* was completely inhibited by the application of n-butanol, demonstrating the strong impact of PA on the JA-signalling pathway in roots.

1. INTRODUCTION

1.1. Rice –A staple food

Oryza sativa is a monocotyledonous angiosperm belonging to the family Poaceae (grass family) and subfamily Oryzoideae (Bajaj and Mohanty 2005). From twenty-three species, only two species have been known for their commercial value being used for cultivation, and these two species are *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice) (Lu *et al.*,1999; Ricepedia, CGIAR, IRRI, Philippines). Cultivated rice (*Oryza sativa*) is one of the most important staple crops in the world and is widely grown in tropical, subtropical, and temperate regions providing 21% of the global caloric intake per capita (Maclean *et al.,* 2002; Gnanamanickam 2009). The cultivated varieties of rice, such as *Oryza sativa* L. and *Oryza glaberrima* have a diploid genome and the chromosome number is n=12.

Oryza sativa comprises two main subspecies: japonica variety also known as sinica, that is sticky, short-grained, and adapted to temperate, whereas indica rice is non-sticky, long grained and adapted to the tropics. A third subspecies, named javanica, and now known as tropical japonica, was identified based on its different morphology such as broad grains, which is cultivated under tropical conditions. Rice does not only have economic importance but also, because of its small genome size of 430Mb, it acts as a monocot model plant to study hormonal signalling pathway, plant-pathogen interaction as well as resistance to environmental stress. In Asia, rice is considered as a first cultivated crop. Around 3000 B.C. preserved rice grains were found in China. Around 1000-750 B.C. paddy grains were found during an excavation in the Hastinapur city of India and considered as the oldest sample in the world.

Apart from being a staple food, rice contains many nutritional properties such as whole grain rice is a good source of protein, carbohydrate, a trace amount of fat, and sodium. Coloured rice serves as sources of antioxidants as well as niacin, thymine, folic acid, and iron (Juliano, 1993; Yawadio *et al.*, 2007; Sulochana *et al.*, 2015; Priya *et al.*, 2019) and has commercial importance in Asia. Rice farming is the major source of income for rural communities, as it is preferably grown in wet regions which are abundant across Asia (Samal *et al*., 2006; Mahapatra and Behera 2011; Arifin *et al*., 2018).

90% of rice is produced alone in the Asian region (Bandumula, N. 2017) **(Figure 1.1)** among which the top 10 rice-producing countries are China, India, Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Philippines, Brazil, and Japan (www.mapsofworld.com).

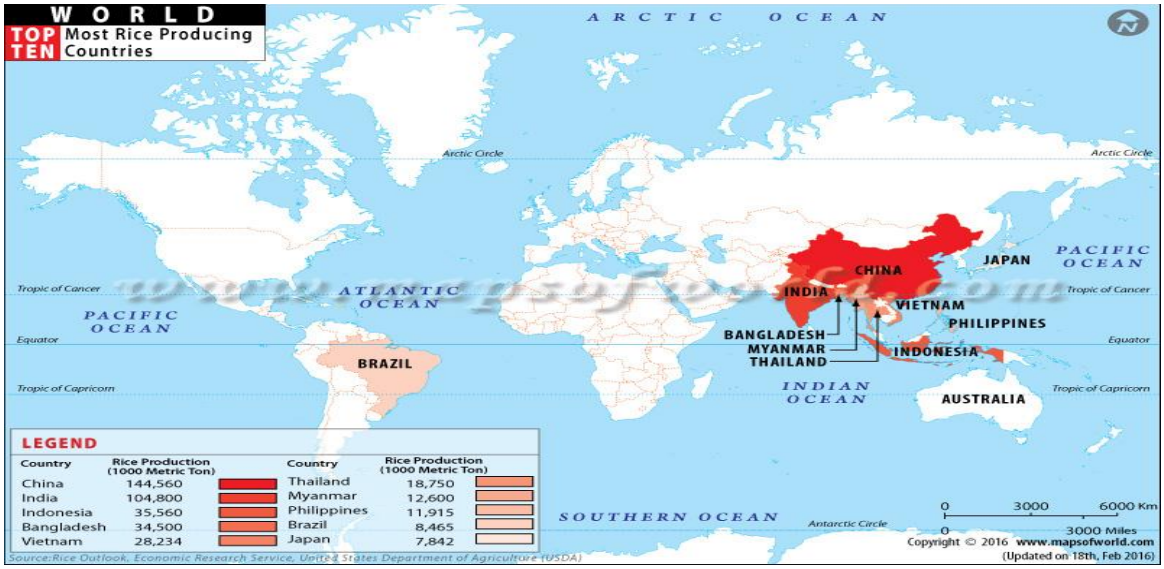

Figure 1.1: Annual production of rice by top ten rice producing countries (www.mapsofworld.com).

As being the staple food for many countries, the demand for rice is increasing rapidly as for the growing population but the area for agricultural land is

limited and it has been reported that by the year of 2050 there will be an increase in rice price by 35 to 37% (IRRI). There will be a reduction in rice yield by 4% in South Asia, 10% in East Asia and the Pacific, and 15% in Sub-Saharan Africa. Crops are exposed to different environmental stress throughout their lifetime and stress is one of the major limiting factors hindering the growth and development of plants. Being originated in the tropical and subtropical region, rice is additionally more sensitive to cold stress compared to other grains that limit the vegetative and reproductive growth (Cruz *et al.,* 2004; Jena *et al.,* 2012; Zhang *et al.,* 2014). Cold stress limits the available geographic land suitable for the cultivation of rice as well as the population is growing rapidly and the lands are limited for rice farming, higher yields will be necessary to fulfil the demand of the growing population therefore the development of stress-tolerant crops is very important (Mahajan and Tuteja 2005).

1.2. Plant stress

Plant stress is defined as an unfavourable condition or factor that alters the plant's metabolism, reproduction, root development, or growth (Lichtenthaler1998; Gaspar *et al.,* 2002). Plant stress is exerted in a variety of forms for varying durations. Some plant stressors are naturally occurring, like cold or drought, while others may be the result of human activity like irrigation and over-urbanization causes root disturbance as the quantity and quality of water and soil changes and result into waterlogging and soil salinization (excess soluble salt or excess exchangeable sodium salt) (Lindahl and Grace 2015; Pereira, A. 2016; Dabi and Khanna 2018).

Stress may be either biotic (when growth and development of plants are altered by living organism, *viz*. bacteria, viruses, nematodes, fungal pathogens

or insect-pest), or abiotic (adversely affecting plant growth and development by the short-term and long-term environmental factors, *e.g.*, drought, light, salinity and cold) (Mahajan and Tuteja 2005; Cramer *et al.*, 2011; Prasch and sonnewald 2013; Pandey *et al*., 2015; Dresselhaus and Hückelhoven 2018; Gull *et al*., 2019; www.waterlog.info). Among abiotic stresses, cold stress has been a major emerging threat for the plants.

1.3. Cold stress as an emerging threat causing plant damage

Cold stress responsibly adds the loss of rice yield. A recent report from IRRI indicates that frequent dropping of temperature contributes to yield loss by 50% (https://www.irri.org/climate-smart-rice). In China, due to cold stress, the recorded yield loss is 3-5 million tons. Korea lost an average yield of 3.9 tons hectare^{-1} in 1980, which is almost more than half of its annual yield hectare^{-1} (https://www.irri.org/climate-smart-rice).

Cold stress is classified into two categories depending on temperature, *i.e.*, chilling stress, and freezing stress. Chilling stress is known at a temperature between 0-15°C, while freezing stress occurs at a temperature below 0°C (Zhu *et al.,* 2007; Chinnusamy *et al.,* 2010). When plants are exposed to temperature stress, it results in the moderation of metabolism in two ways. Initially, plants try their best to adjust their cellular metabolism changes in response to increasing or decreasing temperatures (Kubien *et al.,* 2003; Kotak *et al.,* 2007; Zhu *et al.,* 2007). Cold stress damages or alters the C_3 cycle and hence is responsible for damage to plant's growth and development (Sage and Kubien 2007; Yamori *et al.,* 2013). Temperature stress alters the catalytic property as well as the structure and function of enzymes, causes membrane disruption and disintegration, and changes the structure of membrane transporters for metabolites (Kubien *et al.,* 2003; Nayyar *et al.,* 2005). To cope

up with adverse conditions, the regulatory mechanism of plants gets activated and the formation of normal metabolites takes place, by metabolite flux the altered metabolites get replaced by normal metabolites. Second, in response to temperature stress, the altered metabolites modify to increase or enhance the tolerance mechanism of plants. It has been found that many metabolites are linked to stress-tolerant mechanisms because they are known to involve in inducing stress tolerance in plants (Schwender *et al.*, 2004; Nayyar *et al.*, 2005; Yang *et al.*, 2019). Plants exposed to cold stress show different phenotypic symptoms, such as reduced leaf expansion, wilting, and withering such as yellowing of leaves along with the death of tissue. Cold stress strictly affects the reproductive development of plants by changing the physiological status of anthers, causes damage to the reproductive stage, thereby delaying heading that further leads to the sterility of pollen grain which is one of the major factors responsible for the reduction in rice-grain yield (Kaneda 1974; Gothandam *et al.*, 2007; Suzuki *et al.*, 2008), alters the fertility of flowers, which has been noticed in rice plants during anthesis where the opening of anthers was inhibited and flowers become sterile as a consequence. Hence, male sterility is one of the major reproductive damage to plants that leads to severe agronomic damage to rice production (Nishiyama 1984; Satake and Hayase 1970). The developmental stages from pollen emergence to fertilization are the most vulnerable to low temperatures throughout the life cycle of rice plants. It has been reported cold stress severely damages the pollen development in the young microspore stage. Exposure of rice plants to a low temperature (12°C) for 4 days at the tetrad stage, results in male sterility in 80% of spikelets. The microscopic view of developing rice anthers reveals a lack of success in anther development as one of the possible reasons for male sterility, after low-temperature treatment. The noticed abnormalities are,

inhibition of anther development, cessation of pollen development, anthers persisting within the flowers after anthesis, and partial or no dehiscence. Cytological observation exhibits a dilation of tapetal layers in cold treated rice anthers (Nishiyama 1984; Gothandam *et al.*, 2007). The dilation of the tapetal layer is facilitated by an important augmentation of cytoplasmic organelles such as mitochondria, proplastids, Golgi bodies, and endoplasmic reticulum (Nishiyama 1984). The triphasic cycle of the resistance mechanism of plants against unfavourable conditions is described in **Figure 1.2.**

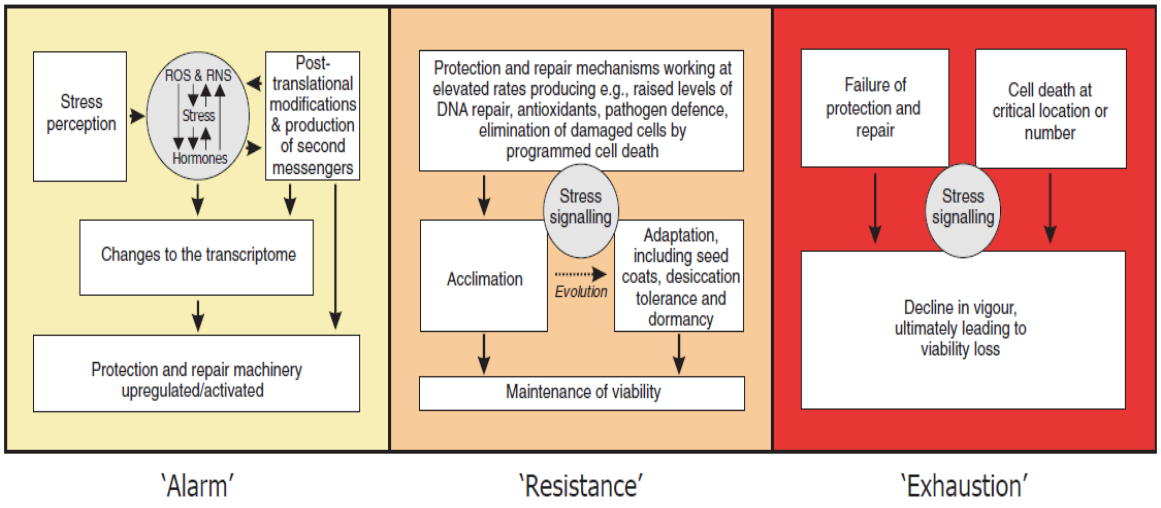

Figure 1.2: General adaptation process of the plant at the molecular level. The 'alarm phase' involves signal perception through ROS (Reactive oxygen species), RNS (Reactive nitrogen species), and hormonal signalling that leads to activation of repair machinery by alterations to the transcriptomes in response to the early damage caused. The 'resistance' phase is reached when there are enough gene products to repair the damage caused by stress which involves the accumulation of antioxidants to protect the macromolecules and activation of PCD to eliminate damaged cells and synthesis of DNA repairs enzymes. The formation of protective seed coats can be seen as a constitutive protection mechanism. The 'exhaustion' phase can be defined as increasing

failure of protection and repair mechanisms when excessive PCD takes place at critical place leads to seed death (Figure from Kranner *et al.,* 2010).

From the past 20 years, various efforts are made to improve cold tolerance in rice which is a very complex process (Maruyama *et al.,*2014). Rice plants need an ideal set of temperatures for their proper growth and development. Different plant species have a different ideal set of temperatures for their proper growth, for example, a set of temperature conditions suitable for one plant but maybe unfavourable for other plants (Kranner *et al.,* 2010). Plants sensitive to cold show structural injuries such as alter the structure and function of cells and tissue and may suffer from metabolic dysfunction under cold stress. Cold, ultimately leads to damage of membrane integrity, leading to solute leakage and loss of compartmentalization, protein assembly, and general metabolic activities (Mahajan and Tuteja 2005). Under optimal temperature or growth condition, ROS such as hydrogen peroxide (H_2O_2), superoxide radical (O^{-}_2), hydroxyl radical (OH), and singlet oxygen ($^1O_2$) is present in low level in the cell but they drastically accumulate in the chloroplast, mitochondria, and peroxisomes under unfavourable or abiotic stress condition like under cold conditions. ROS can damage electron transport chains in both chloroplasts as well as mitochondria (Apel and Hirt 2004; Suzuki and Mittler, 2006; Skopelitis *et al.*, 2006; Mittal *et al.,* 2012; Sharma *et al.,* 2012; Hwang *et al.,* 2019; Janku *et al.,* 2019). Ambient temperature can be sensed by chloroplasts, as a result of which an imbalance occurs between the efficiency to harvest light energy and efficiency to dissipate this energy, this leads to the development of more excitation pressure on PSII, ultimately causes reversible down-regulation of PSII or seize the activity of PSII and disrupts the structure and function of D1 protein (Miura

and Furumoto 2013) as D1 proteins associated with D2 proteins, forms the functional cofactor of PSII and involved in the transformation of light energy by oxidation and reduction of water and plastoquinone respectively to facilitate the photosynthesis. Over reduction of PSII causes the formation of ROS which alters the photosynthetic efficiency of plants or can damage even entire cells (Zhao *et al.,* 2020). ROS causes oxidative degradation of polyunsaturated fatty acids in the result of which there is an accumulation of malonaldehyde which is extremely toxic to the cell and alters the cellular function or causes cellular damage (Sharma *et al.,* 2012; Ayala *et al.,*2014).

1.4. Climate change-Global warming vs cold stress

Global warming is often considered as an outcome of climate change but cold stress is also one of the major threats emerging due to climate change and causes the blurred border of seasons. Climate change occurs due to natural as well as human activity. Natural factors such as continental drift, volcanoes, ocean currents, and human activities such as pollution, urbanization, industrialization, deforestation are the main causes of climate change (Jungclaus *et al.,* 2010). By the year 2039, there will be a reduction in rice yield by 4.5-9% in India (Guiteras 2009). The variable rainfall during monsoon severely affecting the Kharif rice (The crops sown at the beginning of monsoon/rainy season/may end-early June and harvested at the end of monsoon i.e. in October-November is called as Kharif crop) yield as well as cold stress inhibit the seed germination in rabi crop (The crops are sown in winter season/mid-November and harvesting is done in early summer i.e. April-May is called as rabi crop). In the Philippines, with every increase 1ºC of growing temperature leads to a decline in rice productivity by 15% during the dry cropping season (January to April) (Peng *et al.,* 2004). During the dry

season crops, a decrease in rice productivity by 0.12 t/ha is associated with every 1ºC drop of temperature when the temperature falls below 19ºC in the Mekong Delta in Vietnam (Nhan *et al.*, 2011).

1.5. Jasmonic acid (JA) and its role

JA is a phytohormone that is derived from unsaturated fatty acid, linolenic acid, and ubiquitously present in land plants. It was discovered as a secondary metabolite in the essential oil of jasmine flowers in 1960 (Pauwels and Goossens 2011; Campos *et al.*, 2014).

JA plays a diverse role under biotic and abiotic stress conditions. In *A. thaliana*, JA contributes to trichome and stamen developments, vegetative growth, regulates cell cycle, and senescence. In *O. sativa*, JA plays role in seed germination, seedling growth, spikelet formation, regulates cell cycle, senescence, photomorphogenesis, and also flower developments (Svyatyna and Riemann 2012; Dhakarey *et al.*, 2016).

1.5.1. Biosynthesis of JA

JA unsaturated fatty acid linolenic acid serves as a precursor for JA synthesis. It is cleaved by the enzyme lipase from the membrane lipid of the chloroplast **(Figure 1.3).** 18 carbon linolenic acid is converted to 13-hydroperoxy-octadecatrienoic acid (13-HODE) by the action of an enzyme 13-lipoxygenase (13 LOX). Allene oxide synthase (AOS) and allene oxide cyclase (AOC) catalyse the formation of 12-oxo-phytodienoic acid (12-OPDA) from 13-HODE (Li *et al.*, 2005). OPDA is transported from chloroplast to peroxisome by JASSY proteins as it has a channel-like structure which facilitates the transportation, studies revealed that the absence of functional JASSY leads to the deficiency of JA (Guan *et al.*, 2019) and ABC

transporter is known as PXA1, COMATOSE and PED3 involved in the transportation of OPDA to peroxisome where it is reduced by an enzyme OPDA reductase to form 3-oxo-2-(cis-2-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0). OPC-8 is then activated to OPC-8 CoA by an enzyme ACYL-COENZYME A SYNTHASE (Huang *et al.,* 2017; Ruan *et al.,* 2019).

The carboxyl side chain of CoA esters is shortened and undergoes a beta-oxidation cycle by an enzyme acyl-CoA-oxidase (AOX), multifunctional protein (MFP), L-3-Ketoacyl-CoA thiolase (KAT), and 4-coumarate to form jasmonyl CoA. Jasmonyl CoA is converted to cis-7-iso-jasmonic (+) acid by the enzyme Thioesterase (TE).

Alteration in JA biosynthesis leads to abnormal spikelet formation which includes reduced stamen and impaired anther dehiscence. Mutants like *cpm2* and *hebiba* have impaired single copy of the AOC gene, hence they do not form OPDA and JA as a result of which they are male sterile (Riemann *et al.*, 2003; Riemann *et al.,* 2013).

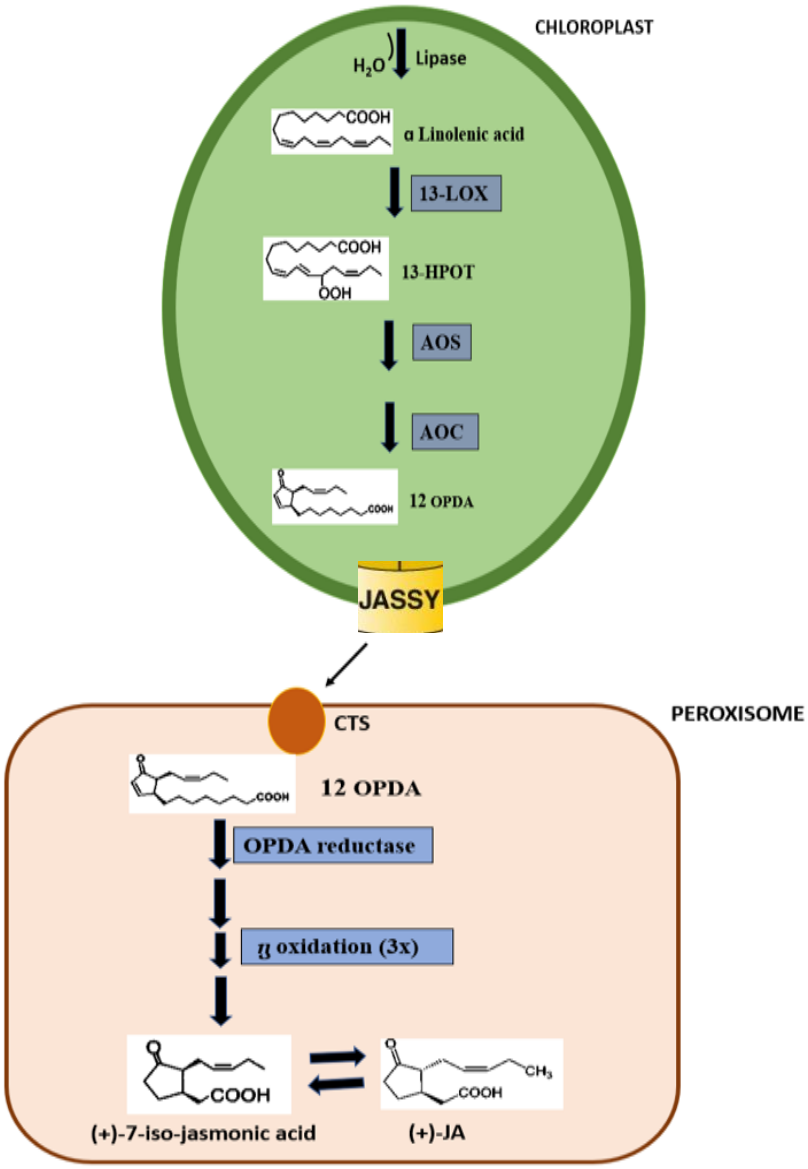

Figure 1.3: Biosynthesis and enzymatic modifications of jasmonic acid. The biosynthesis of JA takes place in the two cell organelles chloroplast (green) and peroxisome (light brown). It is initiated by the enzyme lipases in the plastid that cleaves the membrane lipids to release 12-OPDA with series of intermediates and the enzymes involved (blue boxes). The OPDA is transported into the peroxisome via specific ABC transporter CTS (yellow) (Theodoulou *et al.*, 2005). OPDA undergoes many cycles of beta-oxidation to

reduces its side chain and finally converted into JA. From the peroxisome, the jasmonic acid is released into the cytoplasm. Abbreviations: 13-LOX: 13-Lipoxygenase, 13-HPOT: 13-Hydroperoxylinolenic Acid, AOS: Allene oxide synthase, AOC: Allene oxide cyclase, OPDA: 12-oxo-Phytodienoic acid, OPR: OPDA-Reductase, CTS: COMATOSE, JA: Jasmonic acid, MeJA: Methyl jasmonate. (Figure modified from Dhakarey *et al.*, 2017)

1.5.2. JA signalling

JA conjugates to amino acid isoleucine to form an active compound called JA-Ile and this reaction is catalysed by an enzyme jasmonyl isoleucine synthetase (JAR1), a member of the GH3 gene family (Staswick and Tiryaki 2004; Wasternack and Hause 2013).

Biochemical diversification: It has been reported that depending on the modification in the pentenyl chain, carboxyl-acid group, and pentanone ring, JA can be matabolized into cis-jasmone by decarboxylation of JA, JA-glucosyl ester, 12-O-gulcosyl-JA, methylation by the activity of enzyme called JA-carboxyl methyl transferase to form methyljasmonate (MeJA), conjugate with amino acid isoleusine to give JA-Ile and by hydroxylation form 12-OH-JA (Koch *et al.*, 1997; Li *et al.*, 2018), among which JA-Ile, free JA, cis-jasmone, and MeJA are considered as a major form of bioactive JA (Wasternack *et al.*, 2013).

CORONATINE INSENSITIVE1 (COI1), which is an F-box protein and part of the Skp1/Cullin/F-box protein (SCF) acts as an ubiquitin E3 ligase complex is a receptor for JA-Ile (Xie *et al.*, 1998; Xu *et al.*, 2002; Feng *et al.*, 2003; Chini *et al.*, 2007; Yan *et al.*, 2007). These hormone receptor complex recruits JASMONATE ZIM DOMAIN (JAZ) protein and induces the proteolytic degradation of JAZ in 26S proteasome (Feys *et al.*, 1994; Thines *et al.*, 2007;

Pauwels and Goossens 2011; Wager and Browse 2012; Tian *et al.*, 2019). JAZ acts as a repressor for transcription factor MYC, as a result of degradation MYC transcription factor relieved from the repression of JAZ and as a result of which transcription of early JA responsive gene takes place **(Figure 1.4)**

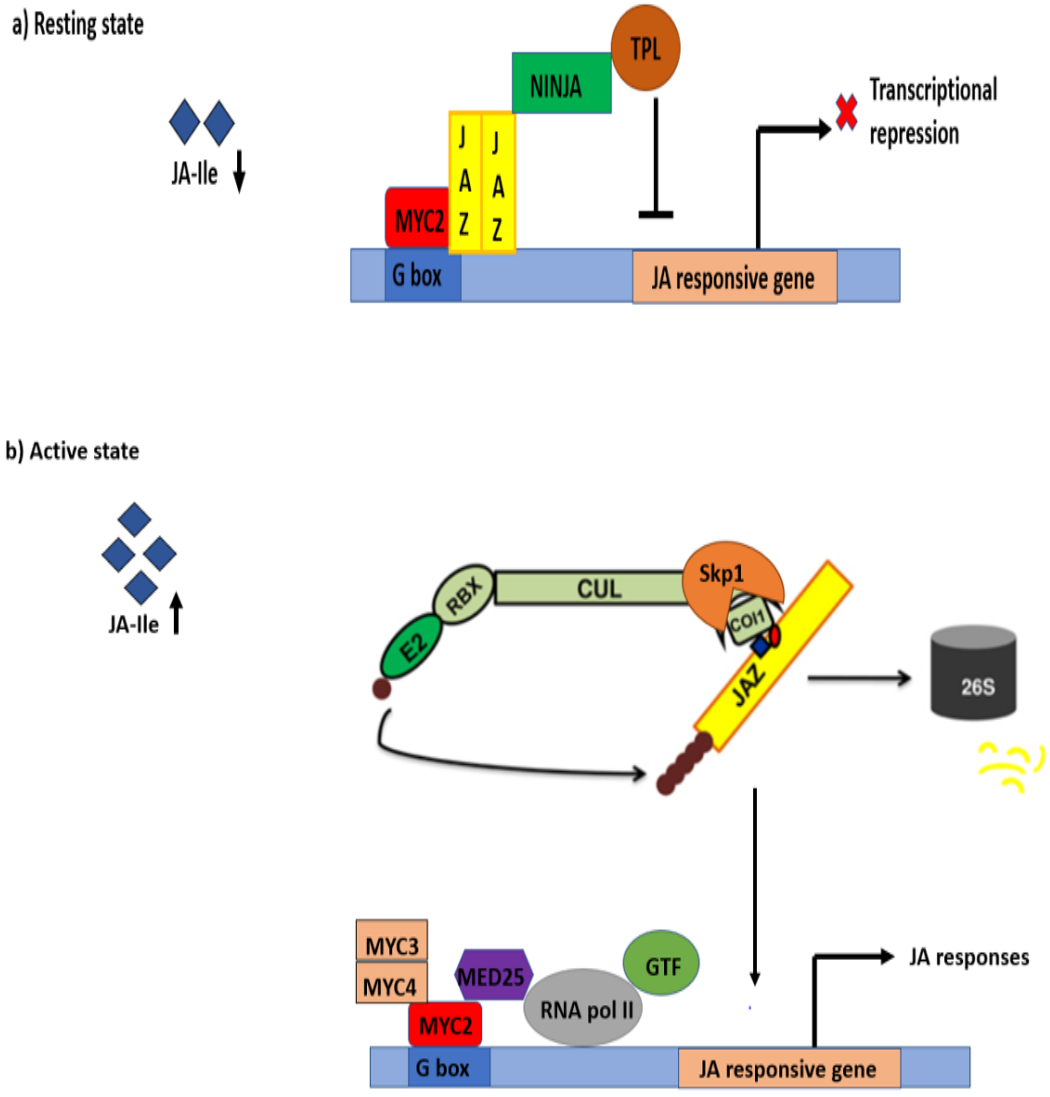

Figure 1.4: JA perception and signalling pathway. a.) When the external stimuli are not present, JA biosynthesis does not take place as a result of which JAZ repressor binds to the transcriptional activator MYC2 and inhibits the

expression of JA mediated genes. JAZ protein binds with the corepressor TPL via NINJA adapter protein. the Corepressor TPL is bound to the JAZ proteins via the NINJA adapter protein and form an active transcriptional repression complex. **b.)** In the presence of stimuli (pathogen/insect/wounding/abiotic stress, rapid synthesis of JA takes place which binds with amino acid Ile. Together with JA-Ile binds with a co-receptor COI1, this hormone-receptor complex recruits JAZ proteins and facilitates the polyubiquitination of JAZ in the 26S proteasome as a result of which MYC2 transcription factor becomes free from repression by JAZ proteins. It then binds to the G-box element present downstream of JA-responsive genes upon homo/heterodimerization. This is followed by the recruitment of MED25 and RNAPol II and general transcription factors as a result of which activation of JA related genes and activation of jasmonate responses. Abbreviations: JA: jasmonic acid, JA-Ile: Jasmonate–isoleucine, JAZ: JASMONATE ZIM-DOMAIN, NINJA: Novel Interactor of JAZ, COI1: CORONATINE INSENSITIVE1, TPL: TOPLESS, CUL: CULLIN1, RBX1: ring box1, Ub: ubiquitin, ASK1: *Arabidopsis* skp1 homolog 1, IP5: inositol pentakisphosphate, GTF: general transcription factor, HDA6, HDA19: histone deacetylase 6,19, MED25: mediator25, RNAPol II: RNA polymerase II. (Figure modified from Sharma and Laxmi 2016).

1.6. Mechanism of Cold tolerance in rice

Changes in ambient temperature affect the cell membrane by changing its fluidity, the structure and function of membrane protein, and osmotic balance, although this process is reversible. But upon long-term exposure to cold temperature, the cell membrane becomes more rigid and enhances the

production of abnormal metabolites, leading to electrolyte leakage, which activates the expression of cold-responsive or cold-tolerance genes.

1.6.1. ABA-dependent pathway

On exposure to cold stress, rice plants accumulate more plant hormone-Abscisic acid (ABA) and stimulate the ABA signalling cascade. The expression of ABA-responsive genes is induced by the ABA-responsive element (ABRE) and the ABRE binding transcription factor (ABF) (Hossain *et al*., 2010). There are 75 NAC genes are present in rice and induced under cold, drought, and osmotic stress, among which *OsNAC6* genes is highly responsive towards cold stress (Ohnishi *et al.,* 2005). The *OsNAC* gene has ABRE in its promoter which is involved in transducing ABA signal, as a result of which it activates and controls the expression of a NAC recognition sequence (NACRS) containing target genes **(Figure 1.5)** to enhance tolerance mechanism in rice against cold (Nakashima *et al*., 2012; Song *et al*., 2011; Zhang *et al*., 2014; Shen *et al.*, 2017).

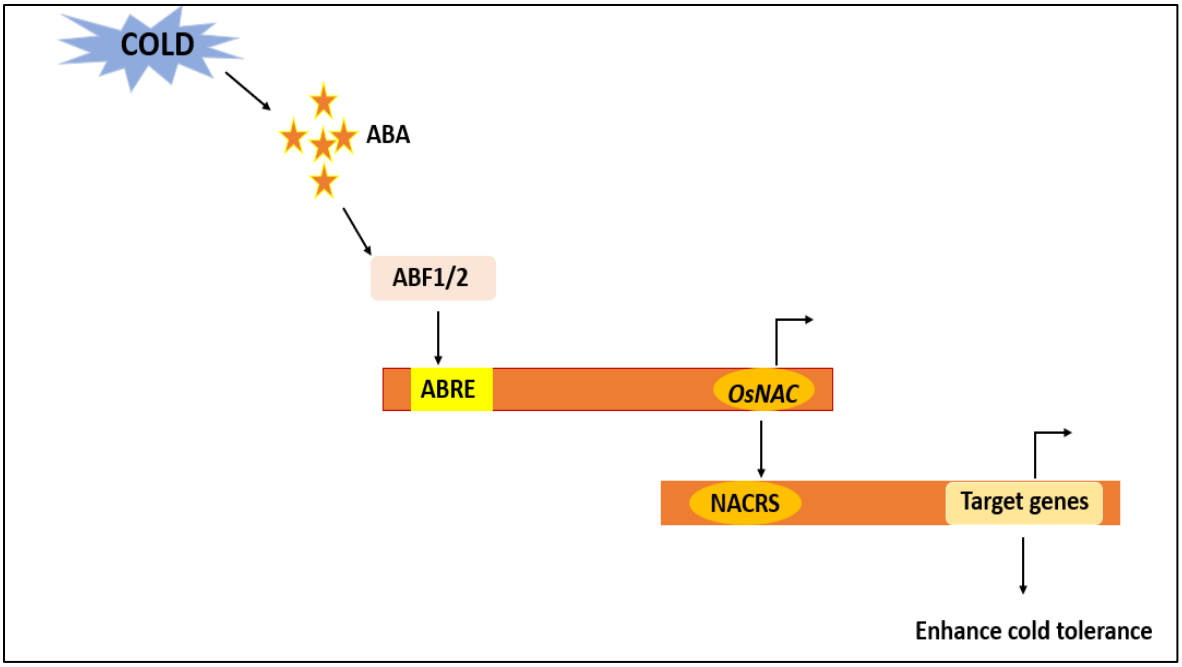

Figure 1.5: ABA-dependent cold signalling pathway. Under cold stress, accumulation of ABA takes place in plants, ABA initiates ABA-signalling cascades by induces the expression of ABF1/2 and ABRE, *OsNAC* genes activates and regulates the expression of NACRS-containing target genes to induce the cold tolerance mechanism in rice. (Figure modified from Zhang *et al.*, 2014). Abbreviation; ABA: Abscisic acid; ABRE: ABA-response element; ABF: ABA-binding transcription factor; OsNAC: NAM-ATAF1,2-CUC2; NACRS: NAC recognition sequence.

1.6.2. ABA-independent DREB-CRT/DRE signalling pathway

Several genes are activated under cold stress upon an exogenous application of ABA, but there are also some genes which not get activated under such treatments, indicating that there are two pathways, *i.e.*, dependent and independent of ABA. (Zhu *et al.*, 2002; Shinozaki *et al.*, 2003; Yamaguchi-Shinozaki and Shinozaki 2006; Thomashow 1999; Chinnusammy *et al.*, 2004). In *O. sativa* there are 163 APETELA2/ethylene-responsive element-

binding protein (AP2/EREBP) genes having double domain called domain a and domain b and a highly conserved AP2/ERF DNA binding domain and are considered as the biggest family of Transcription factor (TF) (Riechmann and Meyerowitz 1998; Huang *et al.,* 2018). They are classified into four subfamilies including AP2, ERF, dehydration-responsive element-binding protein (DREB), and RAV (related to ABI3/VP1) family genes based on their sequence similarity and the number of AP2/ERF(APETELA2/ethylene responsive factor) domains present in encoded proteins (Sakuma *et al.,* 2002; Sharoni *et al.,* 2011). The AP2 subfamily has twenty-four genes considered to encode two complete AP2/ERF domain. In the RAV subfamily, five genes encode a single AP2/ERF domain and a single B3 domain, whereas a large amount of i.e. 134AP2/EREBP genes encoding single AP2/ERF domain has been assigned to DREB and ERF subfamilies. Based on the amino acid sequence similarity of the AP2/ERF domain, 134 genes are again classified into two subfamilies DREB and ERF, of which 57 genes encode DREB/CBF like proteins whereas 77 genes encode ERF like proteins. In all the proteins encoded by ERF subfamilies genes have conserved alanine and aspartic acid at position 14 and 19, respectively, whereas proteins encoded by CBF/DREB subfamilies genes have valine and glutamine at position 14 and 19, respectively (Sakuma *et al.,* 2002; Liu *et al.,* 2006; Sharoni *et al.,* 2010).

Dehydration-responsive element (DRE) is designated as a specific *cis*-element that has conserved 9bp core sequence and was first identified in the promoter region of gene rd29A that is responsive to drought (Yamaguchi-Shinozaki and Shinozaki 1993). DRE is involved in many stress responses via ABA-dependent and ABA-independent signalling pathway (Yamaguchi-Shinozaki and Shinozaki 1994; Liu *et al*., 1998; Kizis and Pages 2002; Dobouzet *et al*., 2003) and it is essential to induce the expression of rd29A

gene on exposure to cold as well as osmotic stress (Ishitani *et al.*, 1998; Saleh *et al*., 2005; Jia *et al.*, 2012).

Subsequently, CRT (C repeat responsive element) has a low temperature-responsive element (LTRE), related to the motif of DRE. It is first identified in the promoter of the cold-inducible gene, such as kin1, kin2, and rab18 in *Arabidopsis* (Kurkela and Borg-Franck 1992; Baker *et al*., 1994 and Ouellet *et al*., 1998; Saleh *et al*., 2005). DRE/CRT responds to cold stress in an ABA-independent manner (Yamaguchi-Shinozaki and Shinozaki 1994; Oh *et al.*, 2005; Saleh *et al.*, 2005). By using yeast one-hybrid screening from *Arabidopsis*, cDNA encoding first DRE binding protein, CBF1/DREB1 (CRT binding factor 1), and DREB2 have been isolated (Stockinger *et al.*, 1997; Liu *et al.*, 1998). *OsDREB1A* genes are induced under cold stress conditions and regulate the cold-signalling pathway independent of ABA (Oh *et al.*, 2005; Agarwal *et al.*, 2006; Garg and Kumar 2016; Deng *et al.*, 2017). DREB1 subgroup has four genes i.e., *OsDREB1A*, *OsDREB1B*, *OsDREB1C*, and *OsDREB1D* was isolated from rice firstly (Dubouzet *et al.*, 2003). *OsDREB1A* is induced under low temperature and binds to CRT/DRE sequence, inducing the expression of genes driven by it. The transgenic rice, overexpressing DREB1A has a function similar to transgenic *Arabidopsis* DREB1A i.e., they are induced under cold stress and improve cold tolerance, thus, indicating DREB1A/CBF cold-signalling pathway is highly conserved in rice (Dubouzet *et al.*, 2003; Ito *et al*., 2006). In rice, overexpression of *OsDREB1A*/CBF leads to cold tolerance by accumulating osmoprotectants and various sugars (Lee *et al.*, 2004; Ito *et al.*, 2006). Under normal conditions, the overexpression of the *OsDREB1A* gene in rice leads to the activation of downstream genes related to cold-tolerance (Oh *et al.*, 2005; Ito *et al.*, 2006).

Upstream of DREB1/CBF a MYC type TF (Transcription factor) controls the expression of DREB1/CBF

A MYC type TF (transcription factor) called ICE1 (Inducer of CBF expression) is present upstream and attach to the promoter region of DREB1/CBF, therefore, activates the expression of DREB1/CBF as a result of which expression of COR genes (cold-responsive elements) is activated and induces the cold tolerance mechanism in *Arabidopsis* (Chinnusamy *et al.,* 2003; Zarka *et al.,* 2003; Toledo *et al.,* 2003 and Novillo *et al.,* 2007). Rice OsICE1/OsICE2 have high similarity in the amino acid sequence to *Arabidopsis* AtICE1/AtICE2. *AtICE1* showed 49.5% similar amino acid sequence to OsICE1, and AtICE2 showed a 48.5 similar amino acid sequence to OsICE2 (Miura *et al.,* 2007; Fursova *et al.,* 2009; Nakamura *et al.,* 2011; Deng *et al.,* 2017). It has been found that overexpression of *OsICE1* and OsICE2 in *Arabidopsis* induces tolerance against cold stress and activates the expression of cold-responsive genes (Deng *et al.,* 2017). ICE1 acts as a master regulator to control the expression of CBF3/DREB1A **(Figure 1.6)**. It has been reported that the expression of 40% COR genes and 46% cold regulated transcription factor genes are controlled by ICE1 (Lee *et al.,* 2005; Chinnusamy *et al.,* 2010).

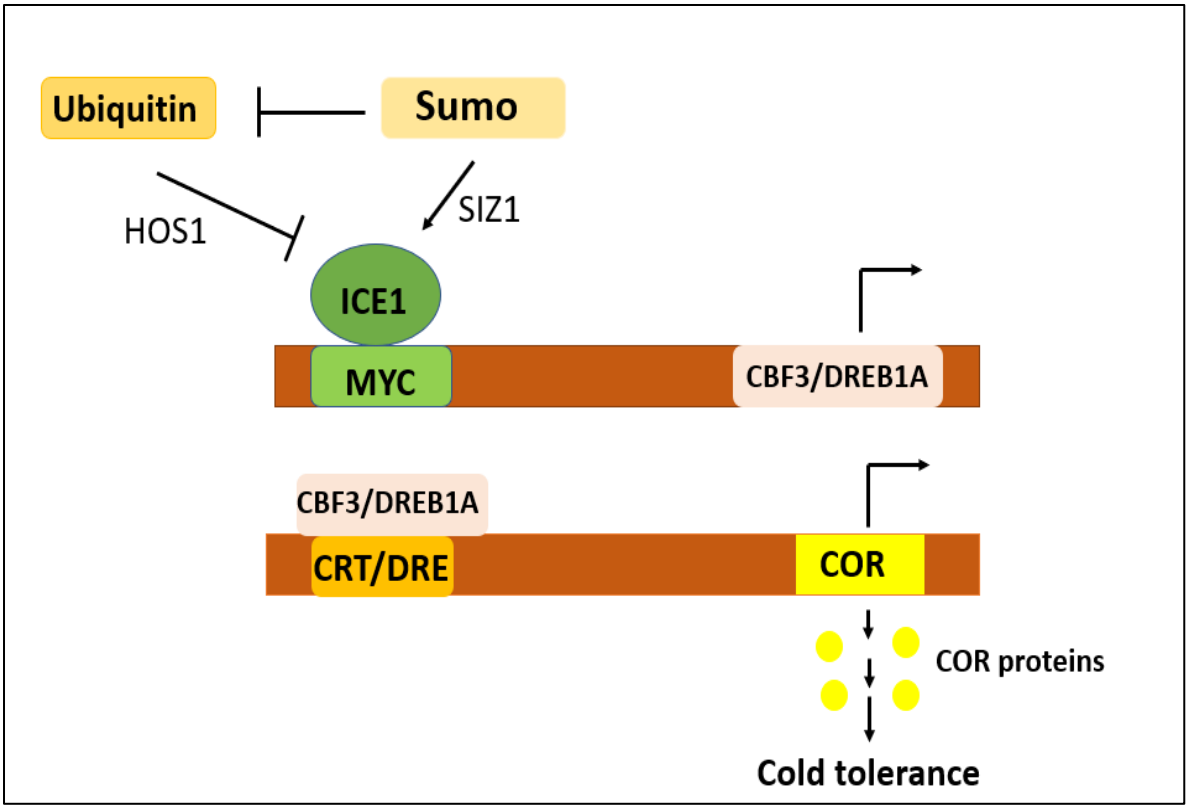

Figure 1.6: Regulation of cold signalling pathway by ICE1 and CBF3/DREB1A. Under the cold stress, TF ICE1 binds to the promoter region of CBF3/DREB1A and induces its expression. CBF3/DREB1A an AP2 type TF binding to the CRT/DRE region and activated the expression of COR. HOS1 is a ubiquitin E3 ligase facilitating the ubiquitylation of ICE1. Its counterplayer SIZ1 facilitates the sumoylation of ICE1, and hence prevents the ubiquitylation and stabilizes ICE1. Abbreviation; ICE1: Inducer of CBF expression, CBF/DREB1A: C-repeat binding factor/ DEHYDRATION RESPONSIVE ELEMENT BINDING protein, CRT: C-repeat responsive elements, DRE: Dehydration responsive element binding, COR: Cold responsive elements. (figure modified from Miura and Furumoto 2013; Pareek *et al.*, 2017).

1.6.3. The secondary messengers sensing cold stress and induction of the cold-signalling pathway.

The accumulation or formation of secondary messengers is triggered by cold stress and various sensors are present which sense these secondary messengers and activate the plant response under cold stress.

1.6.3.1. Role of Ca^{2+} as a secondary messenger to enhance cold tolerance.

When plants experience cold stress the rigidified membrane acts as a signal and stimulates the influx of more cytosolic Ca^{2+} from intracellular calcium stores (Chinnusammy *et al*., 2007). Different types of Ca^{2+} ion sensors are reported which include CaM (Calmodulin) and CMLs (CaM like), CAMTA (CaM binding transcription activator), CDPKs (Calcium-dependent protein kinases), CCaMK (Calcium and Calcium /CAM dependent protein kinases), CBL (Calcineurin B like protein kinase) and CIPK (CBL interacting protein kinase), among which CDPKs work as positive regulators under cold stress (Saijo *et al.*,2000; Townley *et al*., 2002; Doherty *et al.*, 2009). Membrane rigidification, an influx of Ca^{2+} and Ca^{2+} sensors regulate the activity of Mitogen-activated protein (MAP) kinase cascade. They are known to regulate the cold-signalling pathway and cold tolerance plants e.g. in *Arabidopsis* a MAP kinase cascade induces the expression of CBF/DREB1s and enhances the cold tolerance (Sangwan *et al.,* 2002; Teige *et al.,* 2004). In *Arabidopsis*, CIPK3 a member of CBL kinase and a calcium sensor present upstream to the plant hormone abscisic acid (ABA) and cold-responsive genes. CIPK3 gets activated under cold stress and activates the expression of cold-responsive genes, i.e. DREB1A **(Figure 1.7)** indicated that CIPK3 is acting upstream of

DREB1A as well as ABA-responsive genes but downstream of Ca^{2+}-channels, showed that CIPK3 is a molecular link between ABA and stress-responsive genes or CIPK3 act as a crosstalk node between ABA-dependent and ABA-independent pathway as the largely cold-signalling pathway is independent of ABA. (Kim *et al.*, 2003). Cold signal senses and is amplified by these calcium signalling cascade which in return activate dehydration-responsive element-binding protein-C-repeat cis-acting element/dehydration responsive element (DREB-CRT/DRE) pathways and these result in inducing the expression of cold-responsive genes (COR) genes that is the major and most important cold signalling cascade against low-temperature stress (Figure 1.6.3). (Zhu *et al*., 2007; Chinnusammy *et al*., 2007).

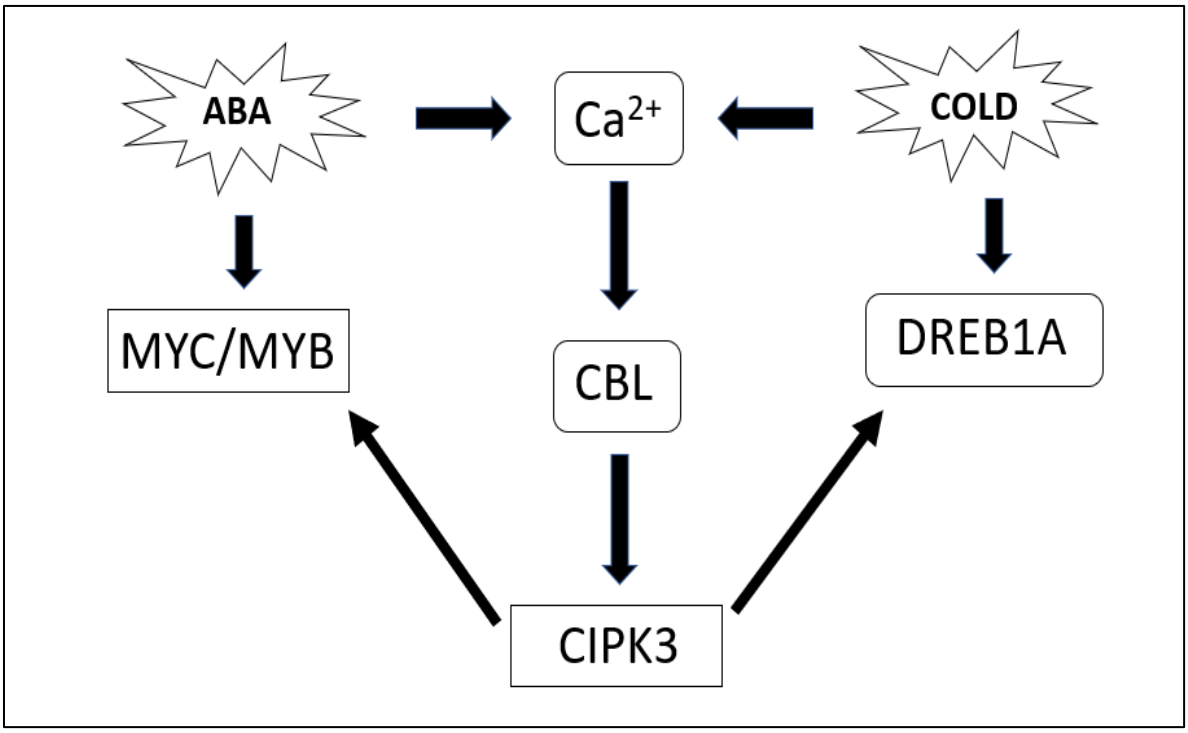

Figure 1.7: CIPK3 is a member of the CBL kinase family. CIPK3 acts as a sensor of Ca^{2+} and positively regulates the expression of ABA-responsive and cold-responsive genes (DREB1A), indicating that CIPK3 acts as a crosstalk

node between ABA-dependent and ABA-independent pathways (Figure modified from Kim *et al.,* 2003).

1.6.3.2. Role of reactive oxygen species (ROS) as a secondary messenger enhancing cold tolerance

In normal conditions plant cellular organelles, *i.e.*, chloroplasts, peroxisomes, and mitochondria contain low amounts of reactive oxygen species (ROS) for example, hydrogen peroxide, superoxide, and free radical as normal by-products of plant cellular metabolism. However, under stress condition (biotic or abiotic) the level of ROS shoot up and they cause cellular oxidative damage when reacting with DNA, protein, and lipid (Apel and Hirt 2004 and Wagner *et al.,* 2014). ROS is an important secondary messenger that is involved in defence signalling pathways under stress conditions. There is coordination between ROS production and its turnover. The level of ROS determines whether it will be destructive for the cell or it will act as a signalling molecule. Under extreme stress conditions, ROS induces a genetically controlled process called programmed cell death (PCD) to eliminate dead and damaged tissues. It has been reported that under cold stress, the cells of rice plants accumulate ROS. They further induce the expression of cold-responsive genes (COR), antioxidants and trigger the cold-tolerance pathway by regulating cold-responsive signalling pathway *via* highly conserved MAPKK and MAPK cascade signalling (Teige *et al.,* 2004; Pitzschke *et al.,* 2006). MAPK ultimately phosphorylates several downstream target transcription factors. At the same time, activation of MAPK cascade signalling takes place that subsequently activates the cold signalling pathway which triggers to promote the formation of COR (cold-responsive proteins). ROS production is important in the plant to activate primary defence mechanism as ROS

involved in the signal transduction pathway to induce cold acclimation (Suzuki and Mittler 2006; Foyer and Noctor *et al*., 2013; Vaahtera *et al*., 2014; Dietz, 2015; Mignolet-Spruyt *et al*., 2016), Also, it has been shown that mutants impaired in ROS production are more sensitive to abiotic stress and also unable to induce systemic signalling against abiotic stress (Mittler 2004; Davletova *et al*.,2005; Suzuki *et al*., 2013).

1.6.4. JA-mediated cold tolerance

Studies revealed that there is crosstalk between the JA signalling component and cold-related transcription factor that is upstream to the cold-responsive gene. Under normal conditions, JAZ interacts and represses the activity of ICE1 **(Figure 1.8).** Upon exposure to cold stress, there is an enhancement in the accumulation of JA that isomerises to an amino acid Isoleucine and form JA-Ile, JA-Ile is recognised by receptor COI1, and this hormone-receptor complex recruits JAZ protein and induces the polyubiquitination and proteasomal degradation of JAZ in 26S proteasome, after this, ICE1 becomes free from the repression of JAZ protein and binds to the promoter of CBF3/DREB1 and induces its expression. Later on, CBF3 protein interacts with CRT/DRE element and induces the expression of the cold-responsive gene to participate in cold tolerance (Thomashow *et al.*, 2010; Hu *et al.*, 2013; Deng *et al.*, 2017).

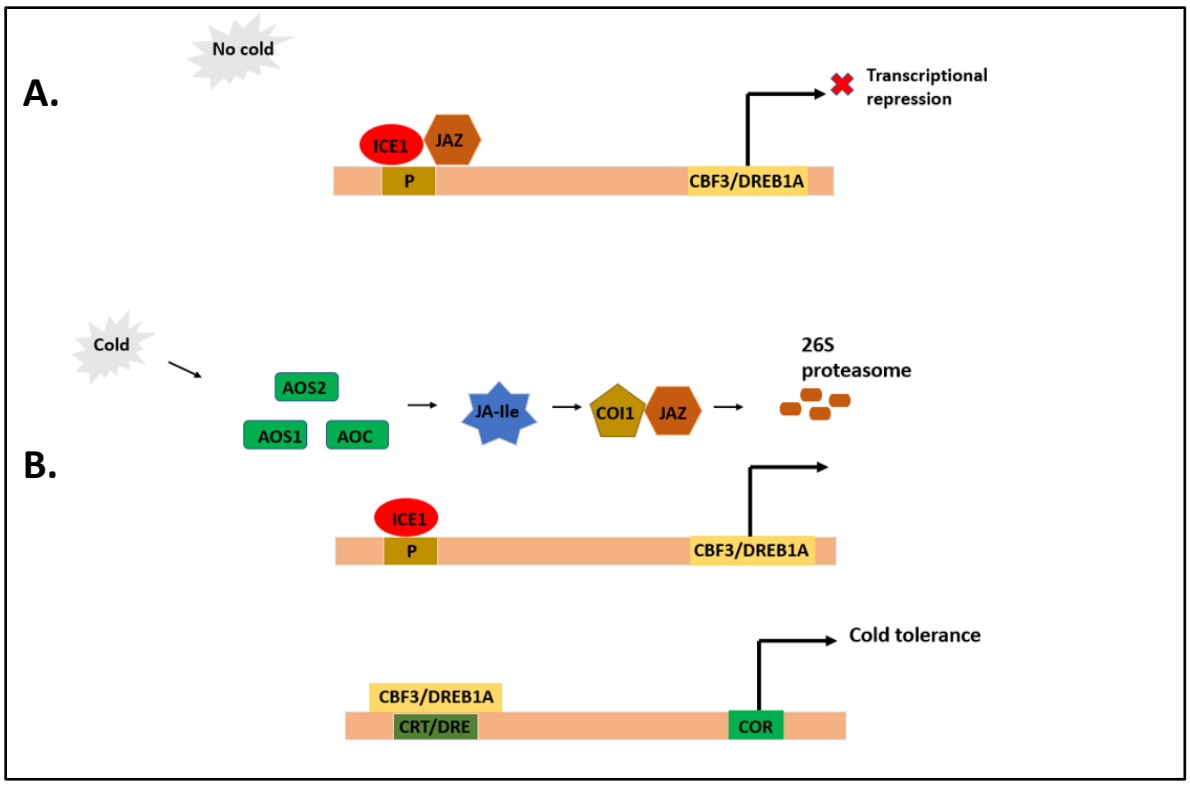

Figure 1.8: JA mediated cold tolerance in plants. (A.) Under normal conditions or in the absence of stress conditions, JAZ proteins act as a repressor and interact with transcription factor ICE1, thus inhibit the CBF3/DREB1A signalling pathway. (**B.)** However, in the presence of cold stress, JA synthesis takes place which binds to amino acid Ile. JA-Ile recruits JAZ proteins and polyubiquitination and proteasomal degradation of JAZ takes place. As a result of which ICE1 is relieved from repression and expression of CBF3/DREB1A takes place. CBF3/DREB1A binds to CRT/DRE and induces the expression of COR genes. Abbreviations: JA: Jasmonic acid, JAZ: Jasmonate ZIM domain, ICE1: Inducer of CBF expression, CBF/DREB1A: (C-repeat binding factor/ DEHYDRATION RESPONSIVE ELEMENT BINDING protein), CRT: C-repeat responsive elements, DRE: Dehydration responsive element binding, COR: Cold responsive (figure modified from Yang *et al.*, 2019).

1.6.5. Accumulation of osmoprotectants and antioxidant enzymes as a known protective mechanism in plants

Osmoprotectants are soluble sugar or compatible sugar which include raffinose, trehalose, sucrose, hexose, glucose, and fructose (Rosa *et al.,* 2009; Yuanyuan *et al*., 2010). Their concentrations shoot up in the cell upon exposure to low temperatures. They help to maintain cell homeostasis as stress alters the composition of the plasma membrane and causes shrink protoplasm due to cold-induced dehydration (Shah and Dubey, 1997; Kandpal and Rao, 1985; Yamazaki *et al.,* 2009) and maintains the oxidative respiration at normal cytosolic pH by quenching the excess H^+ in the cell (Venekamp, 1989). Antioxidant enzymes formed under stress conditions scavenge excess of ROS and protect cells from oxidative damage (Caverzan *et al*.,2016). Non-enzymatic antioxidants include ascorbate (AsA), carotenoids, tocopherol, glutathione (GSH) and phenolic compounds are major cellular redox buffers (Mittler *et al.,* 2004; Scandalios, 2005; Caverzan *et al.,* 2016), although these compounds directly act on ROS and scavenge or reduce them for antioxidant enzymes. Enzymatic antioxidants include peroxidase, catalase, superoxide dismutase, *etc*. (Asada 1999; Mittler 2002; Mittler *et al*., 2004). However, it is currently unknown how these mechanisms are linked to phytohormonal pathways.

1.7. JA crosstalk with other phytohormones

The functions of various plant hormones are overlapping with each other during the growth and development of plants as well as under stress conditions, so it is important to study the crosstalk among different phytohormones to understand the role and responses of hormones in plants. In

addition, many studies revealed Jasmonic acid plays a major role to regulate plant response under environmental stress conditions (biotic and abiotic). Jasmonic acid crosstalk with other plant hormones signalling pathways like auxin, ethylene, cytokinin, abscisic acid (ABA), gibberellic acid (GA), salicylic acid (SA), brassinosteroids and work in a complex signalling pathway to controls the plant growth and developmental process. JA and Auxin signalling pathways regulate the plant growth and development in a coordinate manner. When plants are supplied with exogenous auxin, IAA-ARF signalling is activated, as a result of which synthesis of JA takes place (Robert-Seilaniantz *et al.*, 2011; Huot *et al.*, 2014; Song *et al.*, 2014; Qi *et al.*, 2018; Yang *et al.*, 2019). On the other hand, endogenous JA activates the function of the auxin synthase gene, thus involved in auxin synthesis (Yang *et al.*, 2019). JA and ethylene coordinately regulate the plant defence mechanism against necrotrophic or hemibiotrophic pathogens.

Interaction of JA-SA: JA signalling pathway is known to induce defence against necrotrophic pathogens or herbivores, whereas SA signalling pathway induces defence against biotrophic and hemi-biotrophic pathogens (Bari and Jones 2009; Pieterse *et al.,* 2012; Caarls *et al.*, 2015). But it has been found that in monocots, the *OsAOS2* overexpressing rice plants infected by *M. oryzae* shows upregulation of PR genes and resistance against *M. oryzae* despite it is a hemibiotrophic pathogen (Mei *et al.,* 2006). So, it has been concluded that in some cases the JA signalling pathway induces resistance mechanism against the biotrophic and hemibiotrophic pathogen. In most of the cases, the crosstalk between JA and SA is negative/antagonistic in dicots plants (Koornneef *et al.,* 2008; Robert-Seilaniantz *et al.,* 2011; Caarls *et al.,* 2015). There are many studies suggesting that the JA-SA antagonistic crosstalk is conserved in rice e.g. SA inhibitis the JA-induced expression of rice-root specific pathogenesis-related

protein (RSOsPR10) (which is induced by JA under stress conditions) (Takeuchi *et al.*, 2011). Some studies revealed the common defence system induced against the pathogen in rice by JA and SA-antagonistic interaction **(Figure 1.9),** as under normal condition high endogenous level of SA found which involved in inducing basal defence and when JA-signalling pathway activated, it suppresses the SA-signalling pathway and level of SA will be decreased and therefore JA induces common defence pathway (Tamaoki *et al.*, 2013). SA-JA interaction induces common defence pathway, therefore JA is able to induces defence against biotrophic pathogens. JA is responsible to induce late defence-related gene expression in infected plants, whereas SA induces early defence-related gene expression (Spoel *et al.*, 2007; Tamaoki *et al.*, 2013; Zhang *et al.*, 2017).

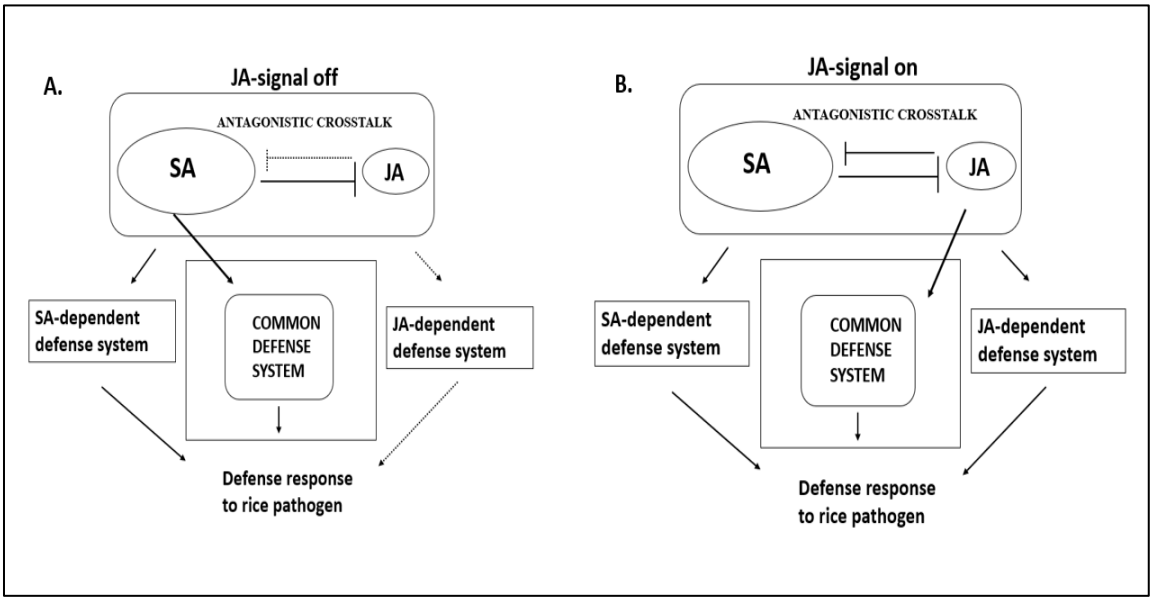

Figure 1.9: In rice, JA and SA induce common defence-system. **(A.)** a common defence system activated when a low concentration of JA is present or when JA-signal is off. **(B.)** a common defence system activated when a high concentration of JA is present or when JA-signal is on. (Figure modified from Tamaoki *et al.*, 2013)

Synergistic interaction between JA-ABA: Some studies have revealed that JA and ABA share the same target and there is extensive crosstalk between both signalling pathways e.g., exogenous application of JA enhances ABA concentration whereas deficiency of JA causes inhibition of ABA accumulation so the crosstalk between JA and ABA- signalling pathway is extensive (Bandurska *et al.,* 2003; De ollas *et al.,* 2013; Puertolas *et al.*, 2013).

The ABA-dependent signalling is controlled by transcription factors, such as ABRE, MYC, and NAC (Zhang *et al.,* 2005; Zhang *et al.,* 2012). Whereas the JA signalling pathway involves the interaction of JA-Ile and COI1 and this hormone-receptor complex involves interaction with JAZ proteins and MYC2 transcription factor which act as a signalling component (Wasternack 2014). The transcription factors JAZ and MYC are involved in the crosstalk between JA-ABA signalling pathways and regulate the growth of plants and defence (Chen *et al.,* 2011). In transgenic plants overexpression of MYC and MYB2 in response to ABA leads to stomatal closure and prevents electrolyte leakages (Abe *et al.*, 1997; Abe *et al.*,2003).

In another example, under drought stress JA-ABA interacts synergistically, under stress condition in *O. sativa* MYC family transcription factor *OsbHLH148* interacts with *OsJAZs*, thus, indicating that activation of MYC transcription factor in response to ABA is highly dependent on JA receptor COI1 and it has been concluded that JA present upstream to ABA (Wasternack 2014; Lorenzo *et al.,* 2004; Seo *et al.*, 2011; Kazan and Manners 2013).

The small GTP binding protein NOG1-2 belongs to the OBG family, NOG1-2 is induced by ABA under abiotic stress conditions and stimulates stomatal closure. In presence of wound or bacterial infection NOG1-2 acts as a positive

regulator and interacts with JAZ9, therefore, NOG1-2 interrupts the interaction of JAZ9 and COI1, as a result of which degradation of JAZ9 does not take place and JAZ9 can inhibit the reopening of stomata as a part of the pathway in ETI (Effector-triggered immunity) indicating that the function of NOG1-2 is JA-dependent. NOG1-2 has GTPase activity and acts as the point of crosstalk between JA and ABA, as under abiotic stress condition, NOG1-2 stimulates ABA-dependent closing of stomata on the other hand under biotic stress NOG1-2 induces closing of stomata as a part of JA-Ile mediated pathway (Ku *et al.,* 2018; Lee *et al.,* 2018).

1.8. Scope of the study

Jasmonic acid is known to regulate the growth and development process in rice plants, also jasmonic acid plays a major role under stress conditions (Syatyna and Riemann, 2012; Liu *et al.,* 2015; Dhakarey *et al.*, 2016). Recent studies revealed that jasmonic acid plays a positive role against biotic stress, it was observed that wild types can produce JA and JA-Ile and also able to produce phytoalexins as a defence mechanism against blast disease, whereas allene oxide cyclase mutants *hebiba* and *cpm2* are JA deficient, and not able to produce phytoalexins. It shows that JA contributing to the formation of phytoalexins (Shimizu *et al.*, 2012). It was reported that allene oxide cyclase mutants of rice i.e. *hebiba* and *cpm2* are JA deficient and are more tolerant against salt stress (abiotic stress) (Hazman *et al.,* 2015). The same phenotypic pattern was found by Kurotani *et al.,* 2015 and it shows that alternation in JA metabolism results in enhanced salt tolerance in rice. But surprisingly the related stresses i.e. salt and osmotic stress showed different results with JA-

deficient mutants. A previous study from our group showed that under osmotic stress conditions jasmonate can be a positive regulator (Tang *et al.,* 2020) or a negative regulator under salinity stress conditions (Hazman *et al.,* 2015; Peethambaran *et al.,* 2018). It has been reported that lack of JA enhanced tolerance against drought which is also among the abiotic stress (Dhakarey *et al.,* 2017). So now there is a question emerging that is the presence or absence of jasmonic acid enhanced tolerance against cold stress? Also, cold stress is one of the emerging threats because of global climate change worldwide, we, therefore, used two AOC mutants *cpm2* and *hebiba* (Riemann *et al.,* 2013) to study the role of jasmonic acid under the cold tolerance mechanism, if it plays a positive role or negative role. These insights can assist us to know more about the function of JA in defence against cold stress. It may also help us to improve the cold tolerance of rice cultivars or the development of rice cultivars which would be tolerant of cold stress.

Considering these points, the main of my thesis was to find out:

1. **What is the role of jasmonic acid in response to cold stress? To study the genes upregulated in response to cold stress**

Oryza sativa L. ssp. japonica cv. Nihonmasari (wild type) and jasmonate-deficient genotype *cpm2* and *hebiba* were available in our lab. It has been reported that on exposure to cold stress, many defence signalling cascades get activated and enhance the cold tolerance in rice. Taking this point into consideration we, therefore, wanted to investigate the role of jasmonic acid in the tissues of root and shoot of control and cold treated seedlings of wild type

and jasmonic acid mutants. We have checked the expression of jasmonic acid synthesis genes, putative upstream signalling genes, and cold-responsive genes to check the coordination between jasmonic acid-responsive genes and cold-responsive genes.

2. **How does cold stress induce morphological, physiological, and biochemical changes in wild type and jasmonic acid mutants on exposure to cold stress**

   As there is a recent report which revealed that jasmonic acid may contribute positively or negatively in plants on exposure to abiotic threats, like under drought and salt stress, JA act as a negative regulator because JA-deficient mutants perform better than the wild type under salt stress whereas under osmotic stress JA acts as a positive regulator as wild type performs better than the JA-deficient mutants. (Hazman *et al.* 2015; Peethambaran *et al.*, 2018; Riemann *et al.*, 2013, 2015; Wu *et al.*, 2015; Tang *et al.*, 2020). So, in our morpho-physiological studies, we wanted to investigate the growth pattern and adaption pattern of wild type and mutants under control and cold treated conditions to see the impact of jasmonic acid. Also, we wanted to investigate how does jasmonic acid contributing to the biochemical levels by measuring the amount of various reactive oxygen species.

3. **How do other plant hormones such as ABA and SA crosstalk with JA in the control and cold treated seedlings on exposure to cold stress**

The JA-signalling pathway has been studied extensively but due to the complex network and crosstalk with various phytohormone signalling pathway, there are limited studies that has been done on its role under various

abiotic and biotic stress. So it is required to unravel the direct influence or role of JA in response to cold stress as well as to assess its interaction with other plant hormones as they provide an opportunity to understand the natural complexity of cold signalling. This information may be useful to enhance crop performance like crop yield under sub-optimal conditions by inducing cold-tolerance mechanisms. In our study, we artificially induced cold stress in wild type and jasmonic acid deficient genotypes and wanted to investigate how does JA signalling cascades activate and interact with other plant hormones to enhance the cold tolerance mechanism of plants. Along with JA, we measured the hormonal quantification of JA-Ile, OPDA, ABA, and SA in cold and control-treated seedling of wild type to unveil the real players involved in cold tolerance mechanism, because in most of the abiotic stress conditions, ABA and SA interact with JA in complex signalling pathway and to control the plant developments and their response towards environmental stress, they interact with each other either antagonistically or synergistically (De Ollas and Dodd 2016; Lee *et al.,* 2017; Lee *et al.,* 2018). So, it is important to unveil or assess the real player as bioactive molecules in these interactions under cold stress.

2. MATERIALS AND METHODS

2.1. Plant material, growth conditions, and stress

Rice (*Oryza sativa* L. *ssp. japonica* cv. Nihonmasari) was used as wild type and the AOC mutants, *i.e.*, *hebiba* and *coleoptile photomorphogenesis 2 (cpm2)* (Riemann *et al*., 2013), were used as jasmonate deficient genotypes. The homozygous mutants seedling was separated based on their large coleoptile length as compared to wild type. In the first step, rice seeds were dehusked, followed by surface sterilization in which they were incubated in 70% ethanol for one minute with subsequent washing of seeds by double distilled water. This step of ethanol and water washing was repeated thrice to ensure proper sterilization of seeds. In the second step of establishing contamination-free seeds, sodium hypochlorite solution was diluted 10-20% of its original concentration and then seeds were soaked for 15-20 minutes, followed by gentle shaking. In the final step, seeds were washed three-four times with sterilized double-distilled water. Seeds were sown in the sterilized magenta box containing 100 ml of 0.4% phytoagar (Duchefa, Netherlands) along with 8% Murashige and Skoog basal salt mixture (MS) medium. The seeds were germinated in a growth chamber (at 25°C, in the light/dark cycle of 12/12h with a light intensity of 120 μmol $m^{-2}s^{-1}$) for 7 days. The temperature of the growth chamber was set to 25°C for control and 6°C for treatment. After 7 days of sowing, seedlings were transferred for adaptation to the cylindrical glass jar with custom-made sterilized floating racks, containing 8% (0.344g/l) of MS media prepared in double-distilled water for 3 days. After 10 days of growth in control temperature, half of the seedlings were exposed to a temperature of 6ºC to induce cold stress while half of the seedlings were left to grow in normal temperature as a control, *i.e*., 25ºC. At 0, 1, 6, and 24 hours

root and shoot samples were harvested separately for control and treated seedlings, immediately frozen in liquid nitrogen and stored in -80ºC until gene expression analysis and hormonal analysis to be done. To obtain the root samples of wild type under control and cold stress conditions treated with n-butanol, seeds are germinated in a growth chamber for 7 days (at 25°C, in light/dark cycle of 12/12h with a light intensity of 120 μmol $m^{-2}s^{-1}$). After 7 days of sowing, seedlings were transferred for adaptation to the cylindrical glass jar with custom-made sterilized floating racks, containing 8% (0.344g/l) of MS media prepared in double-distilled water for 3 days. The roots were treated with 0.5% (v/v) n-butanol for 1 hour except for the control plant which left untreated. After that half of the seedlings were exposed to a temperature of 6°C to induce cold stress while half of the seedlings were left to grow at the normal temperature at 25°C. At 0 and 6 h root samples were harvested separately for control and treated seedlings.

2.2. Analysis of morphology

Seeds were sown in sterilized magenta box containing 0.4% phytoagar medium and germinated for 6 days in the growth chamber under control condition with temperature 25ºC in light/dark cycle of 14/10h with a light intensity of 120 μmol $m^{-2}s^{-1}$. Seedlings were transplanted into a hydroponic system for adaption containing 8% MS medium for one day. New freshly prepared hydroponic systems were precooled at a temperature of 6ºC, at day seven, half of the seedlings were transferred from control to precooled medium and exposed to a temperature of 6ºC for two days to induce cold stress. The length of the shoot and root were measured using a scale with a precision of ±0.5mm. After drying in an oven for 48 hours at 80ºC root and shoot dry weights were measured using a balance with a precision of 0.0001g.

2.3. Measurement of relative water content (RWC) of shoots

RWCs were calculated from the shoot of control and treated plants. The fresh weight of the leaves was determined and then leaves were transferred to the closed 50 ml Cellstar® polypropylene tube containing distilled water. After 12 hours, samples were removed from the tube, gently touched on tissue paper to wipe out excess water present on the leaf surface, and reweighed again to obtain the turgid weight. Thereafter, samples were dried in a hot air oven at 70ºC for 72 hours and the dry weight was determined. RWC was determined using the method proposed by Barrs and Weatherly (1962) with slight modification, and was calculated by using the formula: RWC (%) = [(FW – DW)/(TW-DW)]x100

Where FW is fresh weight, DW is dry weight and TW is the turgid weight.

2.4. Measurement of malondialdehyde (MDA) in roots and shoots

Lipid peroxidation was measured as the amount of MDA determined by the thiobarbituric acid (TBA) reaction, as described by (Heath and Packer, 1968) and (Gallego *et al*., 1996) with minor modification. Leaf (0.1g) and root (0.1g) of control and treated plants were harvested and homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10000 x g for 20 min. From the mixture, 1 ml of resulting supernatant were taken and 1 mL of TCA (20%) containing 0.5% (w/v) of TBA, and 100 µl BHT (4% in ethanol) was added to it. For a blank value, the tissue homogenate was replaced with 1 mL of 3% TCA. This solution was heated at 95°C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10000 x g for 15 min and absorbance was recorded at 532 nm and 600 nm in a UV-Vis

spectrophotometer (Uvikon XS, Goebel Instrumentelle Analytik GmbH, Germany). The blank contained all reagents minus the sample. The MDA concentration was determined by dividing the difference in absorbance (A532 – A600) by using its molar extinction coefficient (155 mM $^{-1}cm^{-1}$), and the level of lipid peroxidation was expressed as mmol g^{-1} fresh weight.

2.5. Estimation of chlorophyll content

Chlorophyll contents were measured from the freshly harvested leaf of control and cold treated seedling by using the method described by Hiscox and Israelstam (1979) with minor modification. Leaf was collected in a sterilized glass test tube containing dimethyl sulfoxide (DMSO). Test tubes were incubated in the oven at a temperature of 60ºC for 1 hour till the complete leaching of pigments takes place. The photosynthetic pigments were then measured at an absorbance of 663 and 645 nm. DMSO was used as a blank. Values of optical densities (OD) were used to calculate the content of chlorophyll ***a*** and chlorophyll ***b*** by using an equation proposed by Maclachlan and Zalik (1963) for chlorophyll ***a*** and Duxbury and Yentsch (1956) for Chlorophyll ***b***.

$$\text{Chl 'a' (mg/g fresh weight)} = \frac{[12.3(OD_{663}) - 0.86(OD_{645})]}{(D*1000*W)} *V$$

$$\text{Chl 'b' (mg/g fresh weight)} = \frac{[19.3(OD_{645}) - 3.60(OD_{663})]}{(D*1000*W)*V}$$

Where, D = Distance travelled by the light path; W = Weight of the leaf material taken; V = Volume of the extract; OD = Optical density.

2.6. Measurement of the endogenous levels of ABA, OPDA, SA, JA-Ile, and JA

Roots and shoots of approximately 120 mg weight were harvested from control cold treated seedlings and the levels of ABA, OPDA, SA, JA, and JA-Ile were quantified by using the standardized technique of ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS) based method according to Balcke *et al*. (2012) by Dr. Bettina Hause (Leibniz Institute of Plant Biochemistry, Halle, Germany). As internal standards [$^2H_5$]OPDA, [$^2H_6$]JA, [$^2H_2$]JA-Ile, and [$^2H_6$] ABA (50 ng each) were added.

2.7. RNA extraction and cDNA synthesis

RNA extraction was performed from the shoot of cold and control-treated seedlings by using the InnuPrep plant RNA kit (Analytika Jena RNA kit) according to the instruction of the manufacturer. We used the steel beads of size 5mm to lyse the cell in the Tissue Lyser (Qiagen). The Spectrum Plant Total RNA kit (Sigma-Aldrich RNA kit) was used to isolate the RNA from the roots of control and cold treated seedlings according to the instructions of the manufacturer. The RNase inhibitor (New England Biolabs; Frankfurt am Main, Germany) was used to prevent the degradation of RNA as RNA is very

sensitive. The M-MuLV cDNA Synthesis Kit (New England Biolabs, Frankfurt am Main, Germany) was used to perform cDNA synthesis from 1µg of mRNA according to the instructions of the manufacturer. The ideal conditions for the synthesis of cDNA are listed in **Table 1.**

Table 1: The reaction program for the synthesis of cDNA and incubation step:

Component	Volume
Total RNA (tempelate)	1 µg
Oligo-dT Primer (40 µM)	2 µl
dNTPs (10mM)	1 µl
Nuclease free water	Up to 16 µl
Incubation: for 5 Minutes at 70°C, centrifuge and placed on ice	
Reverse transcriptase buffer (10x)	2 µl
RNase Inhibitor (10U/µl)	1 µl
M-MuLV Reverse Transcriptase (200 U/µl)	1 µl
Incubation: for 1 hour at 42°C, for 10 minutes at 90°C, storage at -20°C	

2.8. Real-time PCR

We used the CFX96 Touch ™ Real-Time PCR Detection System from Bio-Rad Laboratories GmbH (Munich) to perform the real-time PCR (qPCR). A plate with 96 wells containing the reaction mixture of 20 µl volume was used. The reaction mixtures contained 200 nM of each primer (forward and reverse), 200 µM of each dNTP, 1x GoTaq colorless buffer, 2.5 mM $MgCl_2$, 0.5 U GoTaq polymerase (Promega, Mannheim, Germany), 1x SYBR Green I (Invitrogen, Darmstadt, Germany), and 1 µl of a cDNA template diluted tenfold according to Svyatyna *et al.* 2014). The components are listed in Table 2 along with the program listed in Table 2. The housekeeping gene, GADPH,

and ubiquitin 10 were used as an endogenous control for normalization. For each treatment, three biological replicates were performed. The oligonucleotide sequence of primer for the genes of interest and housekeeping genes are listed in Table3.

Table 2. The concentration of components in the qPCR approach for individual component with a volume of 20 µl:

Components	Volume
cDNA template (1:10)	1 µl
Colorless GoTaq® Reaction buffer (5x)	4 µl
Nuclease free water	12.15 µl
dNTPs (10 mM)	0.4 µl
Forward Primer (20 µM) 0.2 µl	0.2 µl
Reverse Primer (20 µM) 0.2 µl	0.2 µl
$MgCl_2$ (50 mM)	1 µl
GoTaq® DNA Polymerase (5 U/µl)	0.1 µl
SYBR® Green I (10x)	0.95 µl

Table 3. qPCR program:

Reaction step	Temperature (°C)	Time (min:s)	Cycle
Initial denaturation	95	3:00	1
Denaturation	95	0:15	
			40
Annealing and elongation	60	0:40	
Preparation step for the melt curve	95	0:10	1
Preparation step for the melt curve	65	0:31	1
Melting curve (0.5 °C increase per cycle)	65-95	0:05	60

Table 4. The sequence of forward and reverse primers of housekeeping genes used for normalization and sequence of genes of interest.

Gene name	Forward (5'-3' primer)	Reverse (3'-5' primer)	RAP ID
OsDREB1A	TTCTCTCTCTTTTCTGGCTTCCGA	GCTCTGTGTTTGCAACTTGTTC	Os09g0522200
OsOPR7	CTCAACCACCGGTTTCCTCA	CCATGCATCAGTCTGCTCT	Os01g0974000
OsAOS1	CACCGTCACCTCGCTCAAGAAG	ACTCCGTATCCGTACAAGCTGATTG	Os03g0767000
OsAOS2	CTCCGCCGTCAATCGTA	GAGCTGGTTGAGTGGATGAT	Os03g0225900
OsJAZ13	ACACGTCAGCTTTAATCCCATAATT	GAATAATCGTGCACTGTACAAATGC	Os10g0391400
OsPLDalpha1	CCTGAAGACATTGGTGCCCT	TCCACTCTCTGGAACACCCT	Os01g0172400
OsJAZ11	CAGCCTTGCCTACCAGACATG	GACGATCCTGTTCTTCCTCTTCTC	Os03g0180900
OsJAZ12	GCAGCGTTTCCTCCAGAA	CACCGCCGCCTTCTTGTAT	Os10g0392400
OsAOC	TGCCTCAACAACTTCACCAACTA	CACATGCGAGAATTAACACTAAA	Os03g0438100

2.9. Statistical analysis

The significant difference among the data of control and cold treated seedling of wild type and jasmonic acid mutants, i.e. *cpm2* and *hebiba*, were analyzed using the SAS v9.4 software (SAS Institute Inc., Cary, NC, USA). A two-tailed Student's t-test using 95% significance was performed to compare between different treatment. The results which are shown in the graphs are represented as the mean value ± standard error.

3. RESULTS

3.1. Morphological, Physiological and Biochemical analysis of wild type and JA mutant

The external appearance of plant, Relative water content, root and shoot dry weight, root and shoot length, chlorophyll content and malondialdehyde content has been analysed in the wild type and JA-deficient mutants under control and cold-treated conditions.

3.1.1. Morphological phenotypes suggest that JA acts as a positive regulator to induce defence against cold stress

To understand the function of JA for cold tolerance in rice seedlings of the wild type and JA-deficient mutants were exposed to low temperatures and the effect on morphological phenotypes were compared. After 5 days of growth in phytoagar medium, WT, *cpm2,* and *hebiba* were transferred to a hydroponic system for 2 days for a better adaptation. Wild type, *cpm2,* and *hebiba* were transferred to pre-cooled water containing MS media and exposed to 6ºC for 2 days to induce the cold stress whereas control samples were kept at a temperature of 25ºC to maintain ideal growth condition. We observed that wild type performed better under cold stress than *cpm2* and *hebiba*. As wild type was in healthy condition with green and healthy culm, fully expanded tertiary leaf in contrast we have found that *cpm2* and *hebiba* showed complete absence of tertiary leaves (Figure 3.1). From our study, we found that wild type showed more tolerance on exposure to cold stress and defended strongly against cold stress compared to *cpm2* and *hebiba*. Therefore, it can be

concluded that JA acts as a positive regulator against cold stress in rice and plays a positive role in rice to adapt to cold stress.

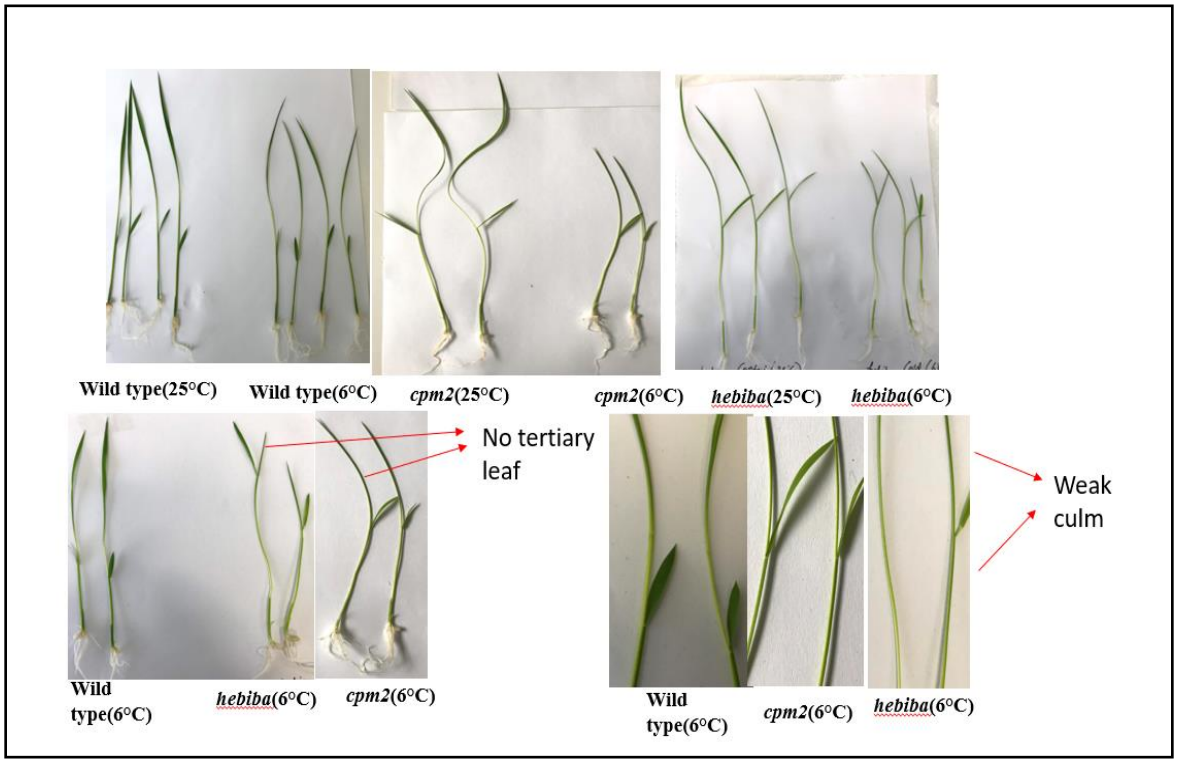

Figure 3.1: Changes in the morphology of wild type, *cpm2,* and *hebiba* under control condition and cod stress exposure. 7 days old seedling of wild type, *cpm2,* and *hebiba* were exposed to cold stress conditions for 2 days, and for control kept at 25°C for the same time.

3.1.2. *Cpm2* and *hebiba* showed more reduction in RWC than the wild type under cold stress

As cold stress often leads to water deficit conditions in the plants, we next compared relative water content (RWC) in wild type and mutant seedlings under control and stress conditions. RWC is correlated with rice performance

under water deficit conditions and extensively applied as a quantitative measure in that context. In our study, we chose RWC as another morphological parameter to study the role of jasmonic acid under cold stress. From our observation, we found that all the cold treated seedlings of wild type, *cpm2* and *hebiba* showed water stress as there is a reduction in the RWC in leaves concerning to their respective controls (Figure 3.2). The wild type showed less reduction in RWC under the cold condition compared with *cpm2*, and *hebiba*. So, from our study on the morphological basis, we concluded that wild type is less sensitive or in other words wild type showed more tolerance against cold stress than *cpm2* and *hebiba.*

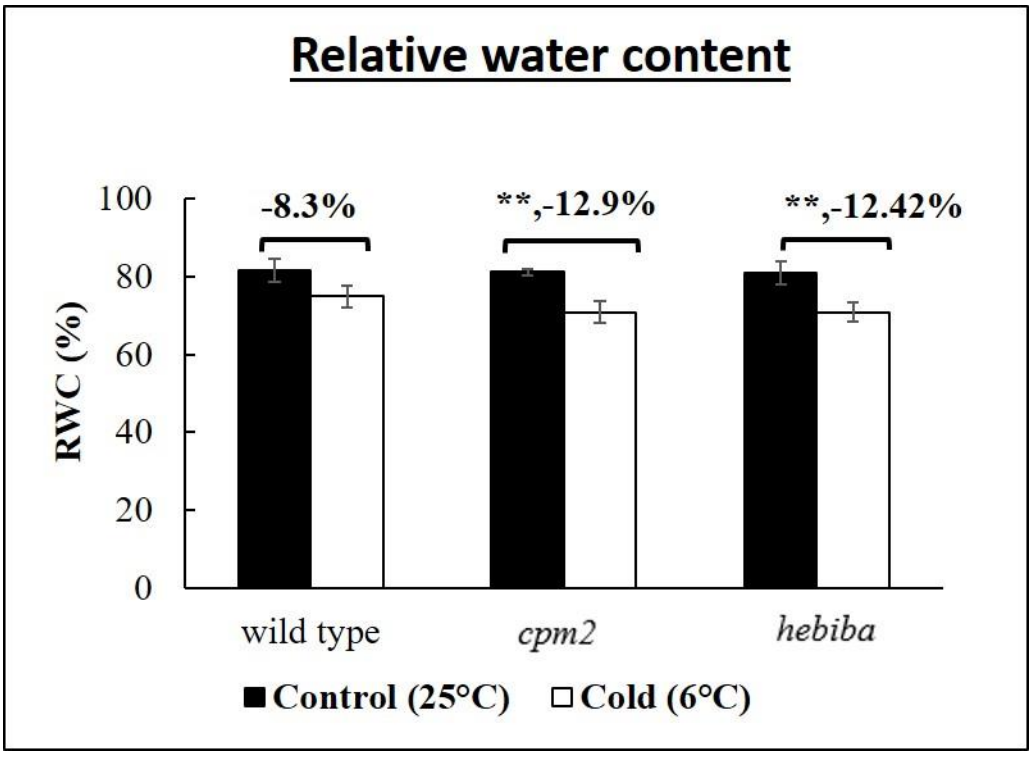

Figure 3.2: The changes in RWC is observed in wild type, *cpm2*, and *hebiba* upon exposure to cold stress (6ºC). The decline in RWC has been observed when 7 days old seedlings of wild type and JA-deficient mutants are exposed to 6ºC

for two days. Values are means ± SE obtained from eleven plants. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; *P < 0.05 and **P < 0.01).

3.1.3. WT showed more accumulation of dry weight with better growth than the *cpm2,* and *hebiba* under cold stress

Growth of the plants was affected severely under cold stress conditions, as wild type, *cpm2,* and *hebiba* showed inhibition of shoot growth on two days exposure to cold with short shoot length compared to their respective control-treated seedlings. There was less accumulation of biomass under cold stress as quantified per dry weight with a significant difference between control and cold treated seedlings (Figure 3.3C and 3.3D). In our analysis, we have found that wild type had the least difference between the shoot dry weight of control and cold treated seedlings compared to *cpm2* and *hebiba.* The cold treated seedlings of wild type had 15% less shoot dry weight compared to control-treated seedlings, whereas in the case of *cpm2* and *hebiba* it was found to be 28 and 23%, respectively (Figure 3.3C). However, in case of roots, we found the same pattern with less accumulation of root dry matter with respect to their control. But in wild type and *cpm2*, the pattern was almost the same whereas the roots of *hebiba* were more sensitive under cold stress and we found *hebiba* had a reduction in dry weight accumulation by 59% compared to its control-treated sample whereas in *cpm2* and wild type it was found to be 31% for both.

The length of the root and shoot were taken as another morphological parameter to analyse the growth pattern and role of jasmonic acid in cold tolerance mechanisms under cold stress conditions. The wild type showed 21% smaller shoot length under the cold condition in comparison with the

shoot of seedlings under control conditions with a significant difference, whereas *cpm2* and *hebiba* showed 38 and 52% reduction in shoot length with respect to their control-treated seedlings respectively (Figure 3.3A). Under cold stress, there was a reduction in the length of roots as well. We found wild type and *cpm2* had a reduction in the length of root 7% and 3.8%, respectively, as compared to their control (Figure 3.3B). Analogous to root dry weight *hebiba* seedlings were affected more than the wild type and *cpm2* and showed significantly smaller root length.

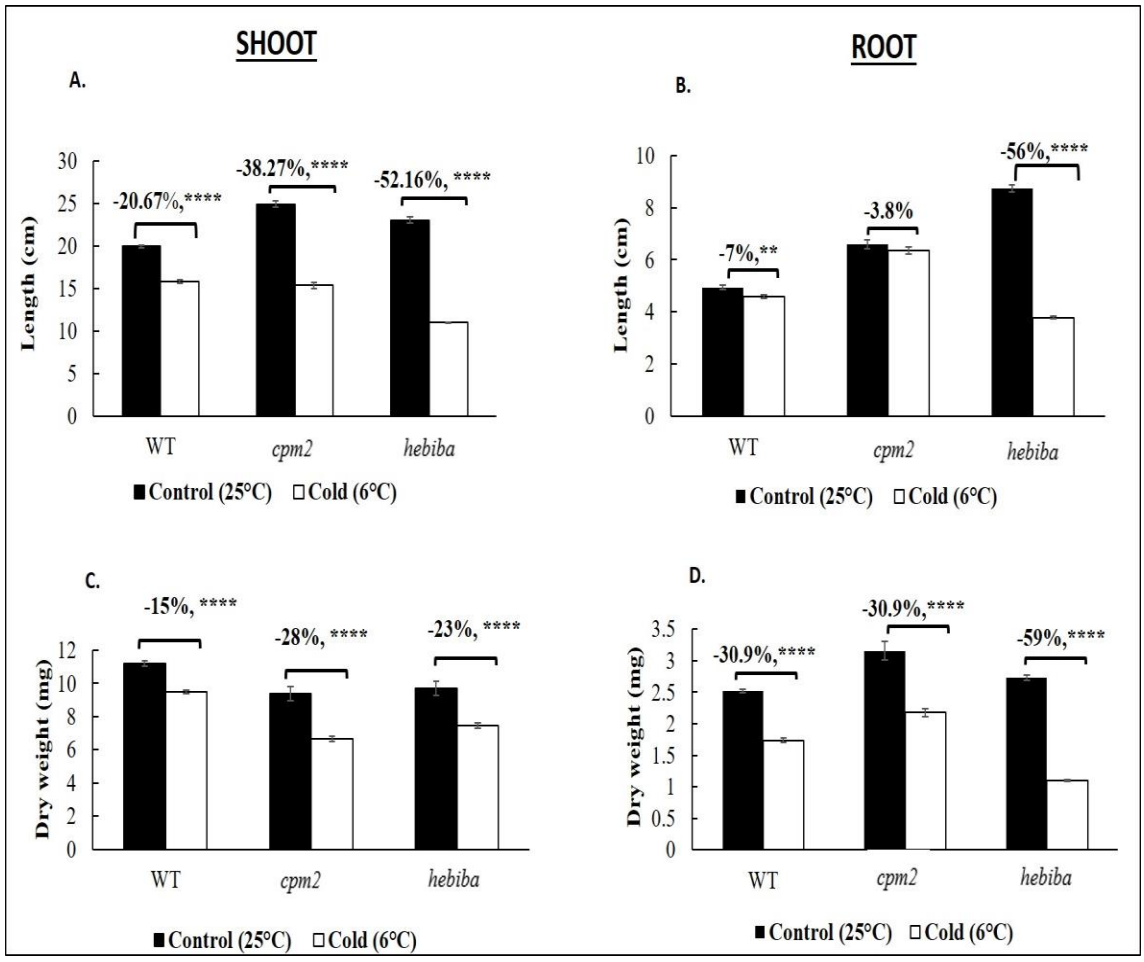

Figure 3.3: Phenotypic changes in the seedlings of wild type, *cpm2*, and *hebiba* on exposure to cold stress. **A.)** Represents the differences in the length

of the shoot of wild type and JA mutants under cold stressed (6ºC) and control conditions (25ºC). **B.)** Represents the differences in the length of the root of the control and cold treated seedlings of wild type and JA mutants. **C.)** Represents the difference in the weight of dry matter in the shoot of wild type and JA mutants with respect to their control-treated root, respectively. **D.)** Represents the differences in the dry weight of cold and control-treated shoots of wild type and JA mutants. Values are means ± SE obtained from nineteen plants. The asterisk represents statistically significant differences between the control and cold treatment (Student's t-test; *P < 0.05, **P < 0.01, ***P ≤ 0.001 and ****P ≤0.0001).

3.1.4. Higher chlorophyll concentration in wild type indicated a positive role of JA under cold stress

Reduction in the levels of chlorophyll pigment (chlorophyll a and chlorophyll b) is a common effect due to abiotic stress and from our result, there is an inverse relationship between cold stress and photosynthetic pigment content. Hence chlorophyll content can be regarded as an indicator of the stress level in plants. On exposure to cold stress of 6ºC, the chlorophyll content tended to decrease in cold treated seedlings of wild type and jasmonic acid-deficient mutants as compared to seedlings which were raised under control conditions (Figure 3.4) Chlorophyll a was present in higher concentration than chlorophyll b in cold and control-treated seedlings of wild type, *cpm2,* and *hebiba.* However, the amount of chlorophyll a tended to decrease on exposure to cold stress in wild type and jasmonic acid-deficient mutants, whereas *cpm2* and *hebiba* were affected more and showed more reduction in the content of chlorophyll a than the wild type. The same pattern was observed for chlorophyll b. Under the control condition, wild type, *cpm2,* and *hebiba* contained more chlorophyll b

content than the cold treated seedlings of wild type, *cpm2*, and *hebiba*. In the control, wild type and *cpm2* have the same amount of chlorophyll content whereas *hebiba* showed less chlorophyll content. Under cold stress conditions, *cpm2* and *hebiba* were affected more and showed a more severe decline in chlorophyll content than the cold treated seedlings of wild type. Under the control conditions, total chlorophyll content was found to be more in wild type than in *cpm2* and *hebiba*. On exposure to cold stress, there was a reduction in the total chlorophyll content, in wild type, *cpm2*, and *hebiba*. The cold treated seedling of JA-deficient mutants affected more than the wild type, as the mutants showed a stronger reduction in the total chlorophyll content as compared to the wild type. In the case of wild type, the reduction in the chlorophyll content was 10%, whereas in the case of *cpm2* and *hebiba* it was 18 and 23%, respectively (Figure 3.4). Comparing all three genotypes, *hebiba* and *cpm2* were responding more sensitive towards cold stress concerning chlorophyll content than the wild type, but among the mutants, i.e. *cpm2* and *hebiba*, *hebiba* was more sensitive to exposure to cold stress than *cpm2*.

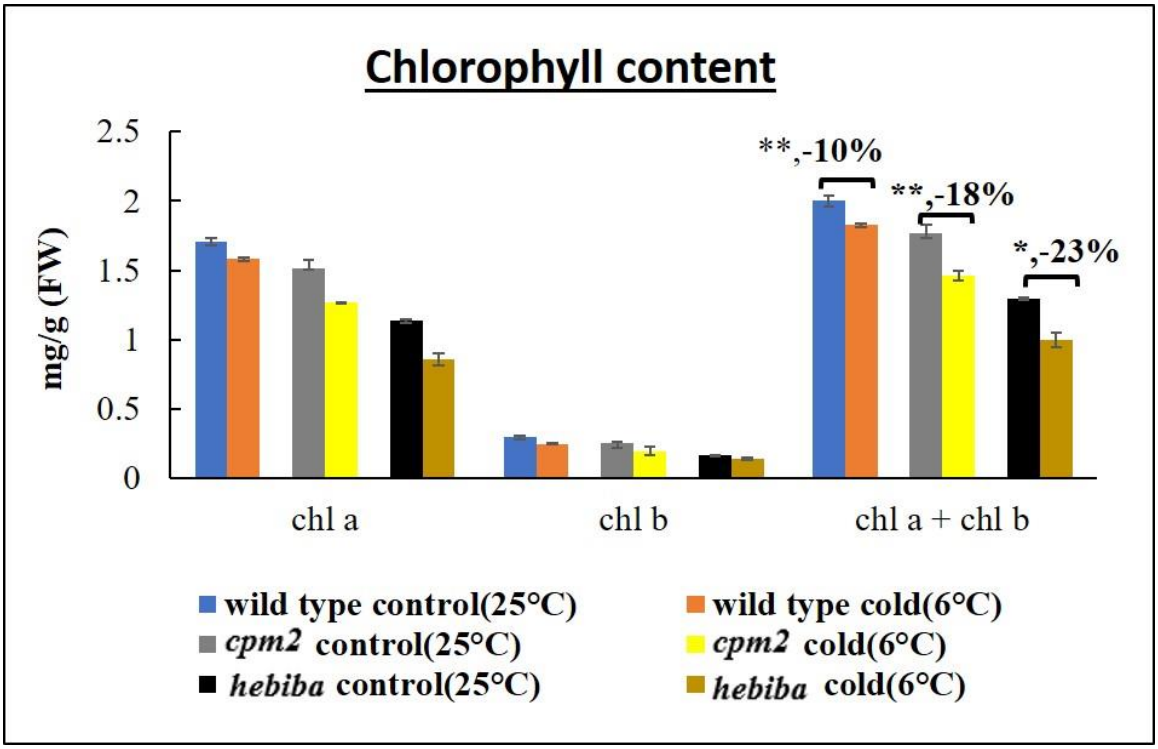

Figure 3.4: Changes in chlorophyll content in the shoot of wild type, *cpm2*, and *hebiba* with respect to their control conditions (25ºC), respectively, upon exposure to cold stress (6ºC) for two days. Values represent the mean of three independent experiments ± SE. The asterisk represents statistically significant differences between the control and cold treatment (Student's t-test; *P < 0.05, **P < 0.01).

3.1.5. JA-deficient mutants *cpm2* and *hebiba* accumulated more MDA compared to wild type

ROS such as hydrogen peroxide, superoxide, and free radicals accumulate under stress conditions and can be used as an additional readout for stress responses. Mostly reactive oxygen species are the by-product of physiological metabolism. The concentration of MDA is considered as a reliable marker of oxidative stress so in our study, we measured MDA. A significant increase of

MDA was found in shoots and roots of cold treated seedlings of wild type, *cpm2,* and *hebiba* (Figure 3.5 A and 3.5 B).

Under control conditions, there was a small amount of MDA found, however, when seedlings were exposed to cold stress, more accumulation of MDA was found in the shoots of wild type, *cpm2*, and *hebiba*. Moreover, wild type showed significantly less accumulation of MDA than jasmonic acid-deficient mutants *cpm2* and *hebiba* upon exposure to cold stress. Cold treated seedlings of wild type showed 140% more accumulation of MDA in the shoot compared to the shoot of the control seedlings whereas in *cpm2* and *hebiba* it was increased to 140 and 141% in comparison to their control respectively (Figure 3.5 A). In the roots, on exposure to cold stress, there is more accumulation of MDA in the wild type, *cpm2*, and *hebiba*. The roots of *cpm2* and *hebiba* were found to be more sensitive towards cold stress than the roots of the wild type as *cpm2* and *hebiba* accumulated more quantity of MDA than the wild type. Under cold stress conditions, the root of wild type accumulated 33% increase of MDA compared to their control, whereas *cpm2* and *hebiba* accumulated 175 and 150%, respectively (Figure 3.5 B).

As after the cold treatment the content of MDA increased, the ROS in our study can be considered a product of oxidative stress and toxic substance that can induce cell damage and death. MDA was found to accumulate more in the jasmonic acid-deficient mutant, i.e. in *cpm2* and *hebiba,* when exposed to cold stress, hence there is an inverse relation between jasmonic acid and ROS. However, it is not clear yet how jasmonic acid eliminates reactive oxygen species.

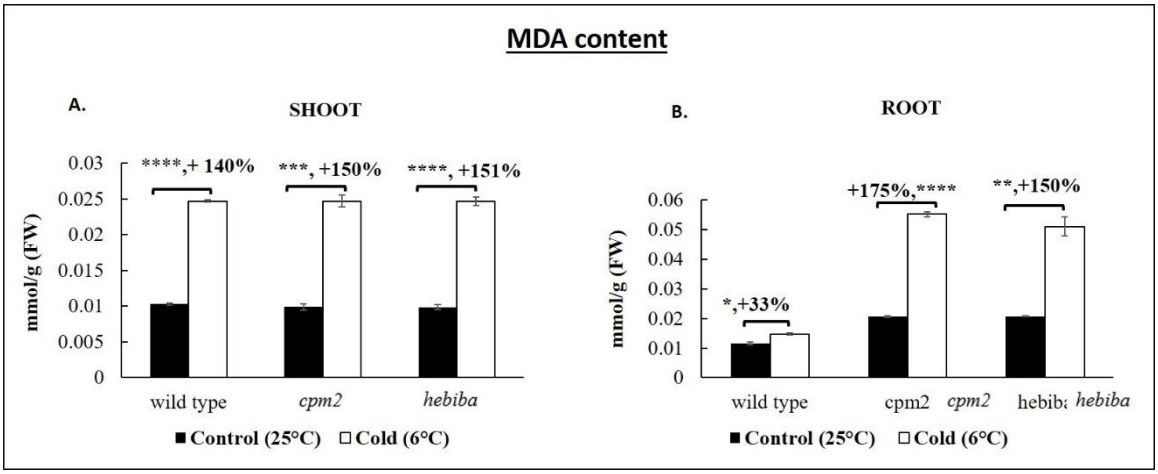

Figure 3.5: MDA levels in wild type and JA-deficient mutants upon cold stress. **(A.)** MDA content in the shoot of wild type, *cpm2,* and *hebiba.* **(B.)** MDA content in the root of wild type, *cpm2,* and *hebiba*. Values represent the mean of three independent experiments ± SE. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; *P < 0.05, **P < 0.01, ***P ≤ 0.001and ****P ≤0.0001).

3.2. Cold stress-induced JA accumulation in rice shoots but not in roots

Being a sessile organism, plants are unable to escape the unfavourable or adverse condition and manifested through alteration in plant growth parameters. To survive, plants must be able to react by producing defence compounds to adapt to a new environment. Up-and downregulation of hormone signalling pathways plays a major role in producing and adapting to a new environment (Benkova *et al.,* 2013). Hence in our experiment, we selected three major plant hormones potentially involved in cold stress tolerance.

As shown in the previous experiments the aerial part of the plant is adversely affected by cold stress in a JA-dependent manner. Therefore, to investigate the

changes in hormonal level the shoot was of major interest in our study. We measured the level of JA as well as its intermediate OPDA and active compound JA-Ile. Other stress-related hormones such as SA and ABA have been measured to investigate their relationship with JA on exposure to cold stress. As the response of hormones is dependent on intensity and time of exposure to cold stress, and the period of response (appearance and disappearance of the active signal) as well as the intensity of the signal received by the cell, we exposed plants for various periods (0,1,6, and 24 hours) to cold. After exposure to cold stress, we found that the level of JA increased after 6 hours of exposure to cold stress and then slightly decreased at 24 hours exposure of cold stress, while there was no significant accumulation of JA in cold treated wild type seedlings after 1 hour of cold stress (Figure 3.6 B). JA-Ile is considered as an active signalling compound and we observed the same pattern as we observed for JA. After 1hour of exposure to cold stress, there was no accumulation of JA-Ile but on exposure to the time duration of 6 hours, there was an accumulation of JA-Ile (Figure 3.6 C) which was decreased at 24 hours. So, we have been found that the 6 hours exposure to cold stress is effective for the synthesis of hormone whereas on the exposure to 24 hours of cold stress JA and JA-Ile decreased to a normal level as compared to control.

OPDA the precursor or intermediate of JA showed an early response after 1 hour (Figure 3.6 A). The amount of OPDA started to decrease after 6 hours of cold treatment and at 24 hours OPDA decreased to control levels. Hence OPDA accumulation precedes that of JA and JA-Ile, as expected for a precursor of these compounds.

ABA is considered as stress-related hormones and has a significant role under abiotic and biotic stress. It is involved in the activation of many ABA-dependent genes under stress conditions. To elucidate the function and mechanism of ABA

under cold stress we considered the measurement of ABA in cold treated wild type seedlings. In the cold stress regime applied in our study, there was no accumulation of ABA (Figure 3.6 D).

Like ABA, SA is another plant hormone. SA is known to have a significant role against many abiotic stresses such as cold, salt, drought, etc. It has been reported that the exogenous as well as the endogenous application of SA enhances the cold tolerance capacity in plants by regulating ROS and inducing the activities of antioxidant enzymes (Salih *et al.,* 2016). We, therefore, included SA in our analysis. In our study, SA showed an interesting temporal pattern in the control plants (Figure 3.6. E). During our experiment, we observed a developmental decrease of SA at 6 hours which recovered almost completely at 24 hours. Under cold stress, such a decrease of SA amounts at 6 hours was not observed, but it remained at the same level as the control. Hence SA might support the plant in coping with cold stress by maintaining is level high under cold stress.

However, in roots the results were different. When we exposed plants for various periods (0,1,6, and 24 hours) to cold, we found there was no accumulation of JA and JA-Ile (Figure 3.7 B and 3.7 C). At 24 hours of cold exposure, JA and JA-Ile remained at the same level as the control, whereas OPDA showed a late response after 24 hours (Figure 3.7 A).

During our experiment, we observed no accumulation of ABA at 1 hour. Unlike in the case of shoot, in root ABA showed a late response. ABA started to recover at 6 hours and almost completely recovered at 24 hours (Figure 3.7 D). In the case of SA, there was no accumulation of SA under cold stress conditions (Figure 3.7 E). Hence, OPDA and ABA showed a late response in case of root under cold stress conditions.

WILD TYPE SHOOT

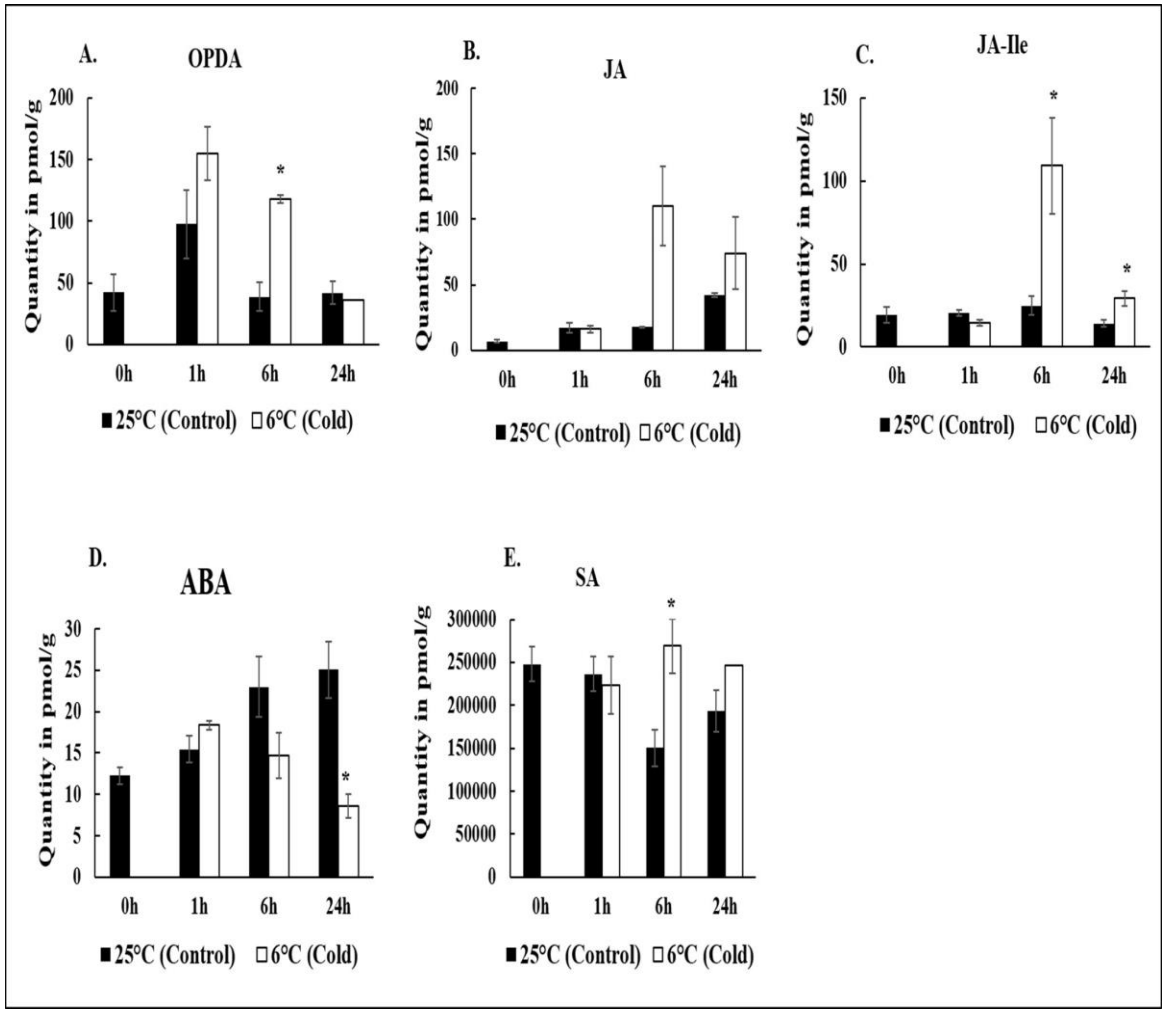

Figure 3.6: Measurement of phytohormones level in the shoot of wild type under control (25ºC) and cold (6ºC) at 0,1,6, and 24 hours of time duration. **(A.)** OPDA in the shoot. **(B.)** JA in the shoot. **(C.)** JA-Ile in the shoot. **(D.)** ABA in the shoot. **(E.)** SA in the shoot. Values represent the mean of three independent experiments ± SE. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; *P < 0.05). Results for the control and treatment are indicated by black and white bars, respectively.

WILD TYPE ROOT

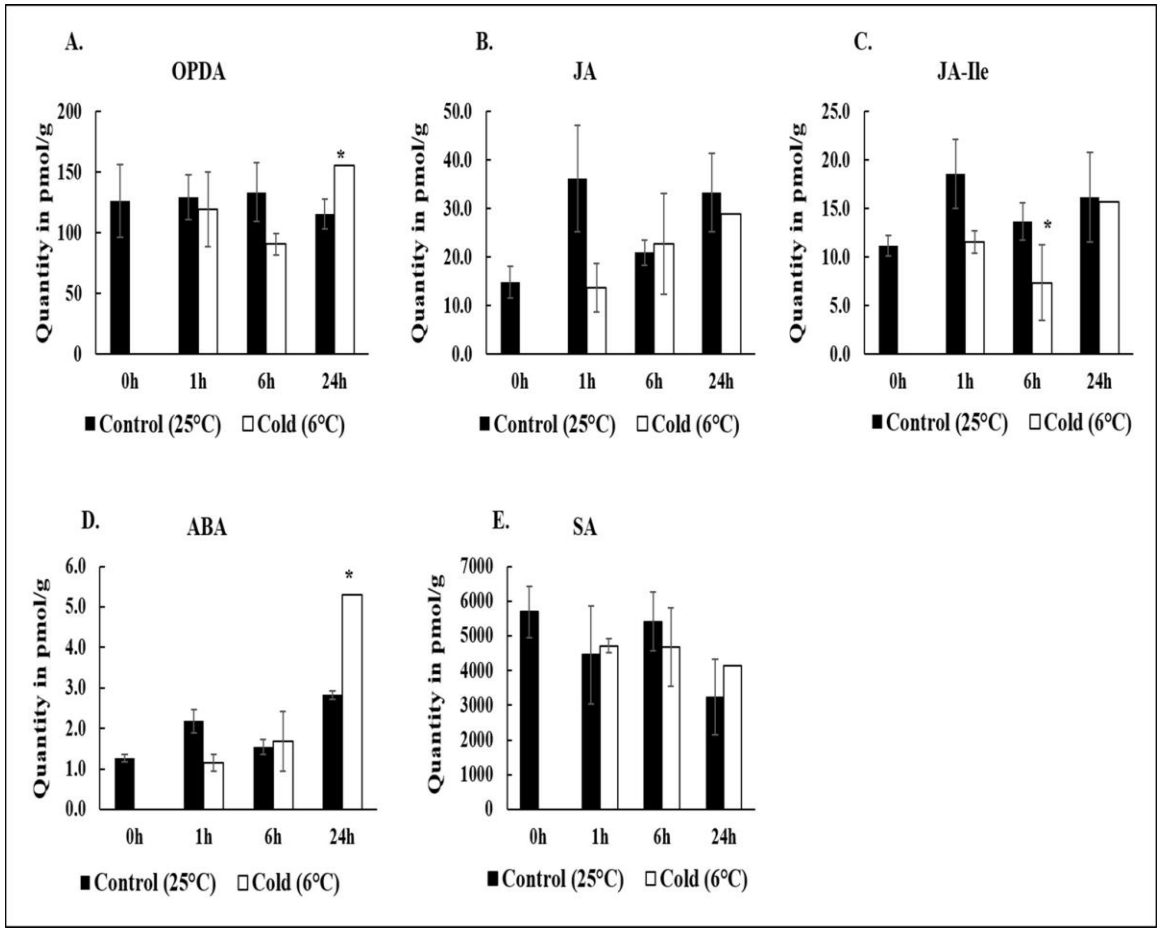

Figure 3.7: Measurement of phytohormones level in the root of wild type under control (25ºC) and cold (6ºC) at 0,1,6, and 24 hours of time duration. **(A.)** OPDA in the root. **(B.)** JA in the root. **(C.)** JA-Ile in the root. **(D.)** ABA in the root. **(E.)** SA in the root. Values represent the mean of three independent experiments ± SE. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; *P < 0.05). Results for the control and treatment are indicated by black and white bars, respectively.

3.3. Transcriptional regulation in response to cold stress

Cold stress regulates many genes. Cold-induced genes are activated by transcriptional activators that are required for cold tolerance. Genes are required for the adaptation process in plants therefore in our experiment we studied selected genes including JA-responsive genes, putative upstream signalling genes, and known cold-responsive genes to identify their significant role in cold stress and JA-dependency. In our study, all the expression data were referred to a wild type sample collected at the start of the experiment (0 h) which served as a calibrator.

3.3.1. *OsAOS2* and *OsOPR7* - two JA-biosynthesis genes with contrasting regulating in response to cold stress

In the JA biosynthesis pathway, ALLENE OXIDE SYNTHASE (AOS) is an enzyme that catalyses one of the first steps in JA biosynthesis in the chloroplast from lipoxygenase-derived hydroperoxides of free fatty acids. It catalyses the step upstream of AOC. In our study, we found *OsAOS2* to be a cold-inducible gene. In wild type shoot, transcripts were induced after 1 hour and accumulated very strongly after 6 hours of cold stress and the expression level remained almost the same after 24 hours of cold stress. (Figure 3.8 A). On exposure to 6 hours of cold stress, wild type and *cpm2* showed an equal levels of gene expression in the shoot, *OsAOS2* being strongly induced, whereas the expression level was weak or almost the same to control in *hebiba* (Figure 3.8 D). In the case of wild type root, transcripts were induced after 1 hour and after 6 hours of exposure to cold stress, the root of wild type, *cpm2,* and *hebiba* showed upregulation in the gene expression with almost the same amplitude (Figure 3.9 D). In the case of wild type root, the strongest expression level of *OsAOS2* was recorded at 24 hours of cold exposure (Figure 3.9 A) whereas in the shoot of the

wild type at 6 hours exposure of cold treatment strongest expression level was recorded, which remained same at 24 hours of cold exposure (Figure 3.8 A). This suggests that the expression of *OsAOS2* is responsive towards cold stress and expression is faster in shoot than the root of wild type. As we found the strong induction of *OsAOS2* in shoot and root of wild type as well as in shoot of *cpm2* and root of *cpm2* and *hebiba* both. Therefore, we concluded, the induction of *OsAOS2* is independent of JA.

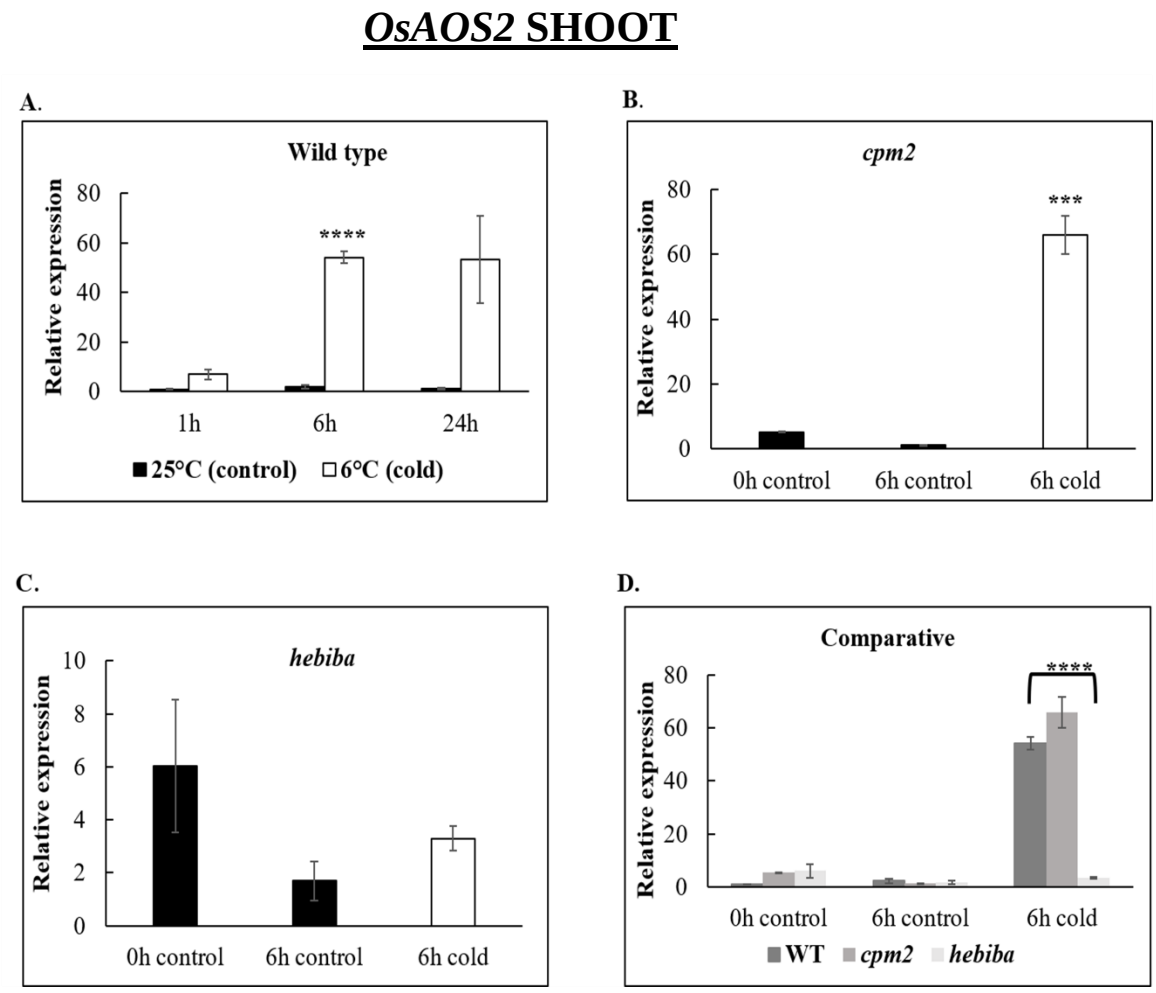

Figure 3.8: Relative gene expression analysis of JA-biosynthesis gene *OsAOS2* in the shoot of wild type and JA-deficient mutants *cpm2* and *hebiba* under control

(25ºC) and cold (6ºC) treatment conditions considered the time duration of 0,1,6, and 24 hours for wild type whereas 0 and 6 hours for *cpm2* and *hebiba*. **(A.)** wild type shoot *OsAOS2*. **(B.)** *cpm2* Shoot *OsAOS2*. **(C.)** *hebiba* shoot *OsAOS2*. **(D.)** shoot comparative graph *OsAOS2*. Data represent the average of three biological replicates with three technical replicates in each experiment. Error bars show the standard error value. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; ***P ≤ 0.001and ****P ≤0.0001).

***OsAOS2* ROOT**

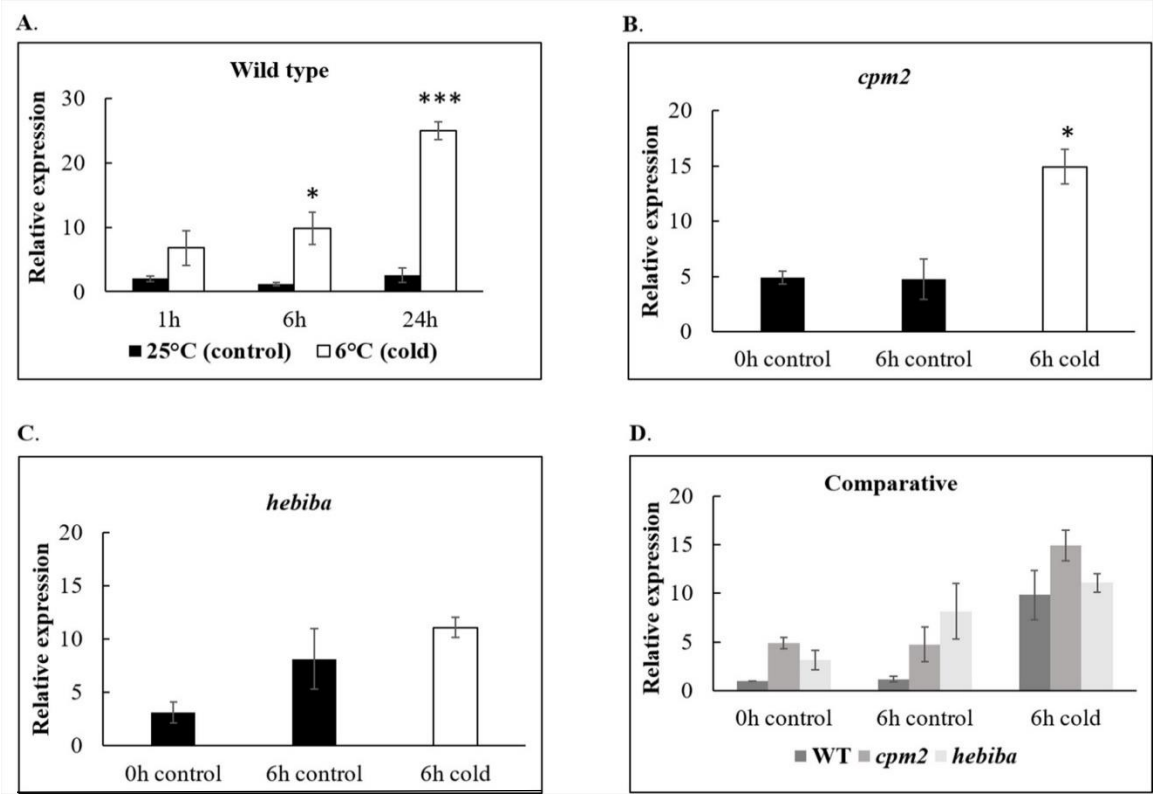

Figure 3.9: Relative gene expression analysis of JA-biosynthesis gene *OsAOS2* in the root of wild type and JA-deficient mutants *cpm2* and *hebiba* under control (25ºC) and cold (6ºC) treatment conditions considered the time duration of 0,1,6, and 24 hours for wild type whereas 0 and 6 hours for *cpm2* and *hebiba*. **(A.)** wild

type root *OsAOS2*. **(B.)** *cpm2* root *OsAOS2*. **(C.)** *hebiba* root *OsAOS2*. **(D.)** root comparative graph *OsAOS2*. Data represent the average of three biological replicates with three technical replicates in each experiment. Error bars show the standard error value. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; *P <0.05, ***P ≤ 0.00.

12- oxo-phytodienoic acid (OPDA) is formed in chloroplasts by tight interaction of AOS and ALLENE OXIDE CYCLASE (AOC) and needs to be transported to peroxisome where it will be catalysed by the enzyme OPDA-REDUCTASE (OPR) and subsequently further converted into JA in several enzymatic steps. *OsOPR7* is a rice OPR which has been demonstrated to have the required substrate specificity (Tani *et al.,* 2008). Hence in our study, we elucidated the role of *OsOPR7* under cold stress in WT, *cpm2*, and *hebiba* root and shoot. The induction of *OsOPR7* was weaker than *OsAOS2* in all genotypes.

On exposure to cold stress of 6 hours wild type shoot showed upregulation in the expression of *OsOPR7* (Figure 3.10 A.), whereas in *cpm2* there was no significant upregulation in the expression of *OsOPR7* found (Figure 3.10 B.). However, *hebiba* showed a very weak upregulation of *OsOPR7* (Figure 3.10 C). The upregulation of gene expression in wild type shoot and root observed from 1 hour of cold exposure, increased in 6 hours exposure, and the highest was observed in 24 hours of cold exposure (Figure 3.10 A and 3.11 A). In the case of root, on exposure to cold stress of 6 hours, wild type showed the highest accumulation of transcripts than the *hebiba* and *cpm2*, respectively (Figure 3.11 D). The expression level JA synthesis gene *OsAOS2* located upstream of AOC was found to be stronger than the JA synthesis gene located downstream of AOC i.e. *OsOPR7*.

OsOPR7 SHOOT

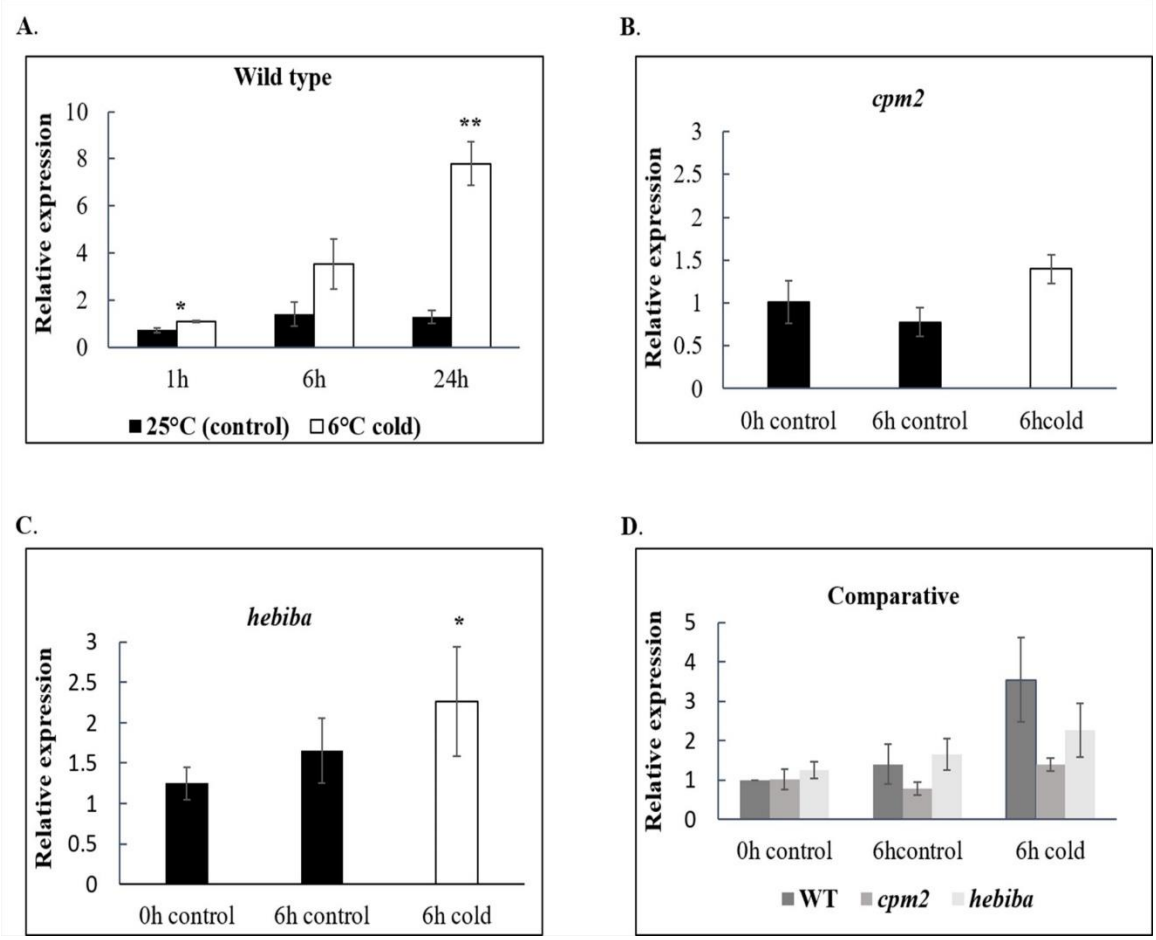

Figure 3.10: Relative gene expression analysis of JA-biosynthesis gene *OsOPR7* in the shoot of wild type and JA-deficient mutants *cpm2* and *hebiba* under control (25ºC) and cold (6ºC) treatment conditions considered the time duration of 0,1,6, and 24 hours for wild type whereas 0 and 6 hours for *cpm2* and *hebiba*. **(A.)** wild type shoot *OsOPR7*. **(B.)** *cpm2* Shoot *OsOPR7*. **(C.)** *hebiba* shoot *OsOPR7*. **(D.)** shoot comparative graph *OsOPR7*. Data represent the average of three biological replicates with three technical replicates in each experiment. Error bars show the standard error value. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; *P < 0.05 and **P < 0

***OsOPR7* ROOT**

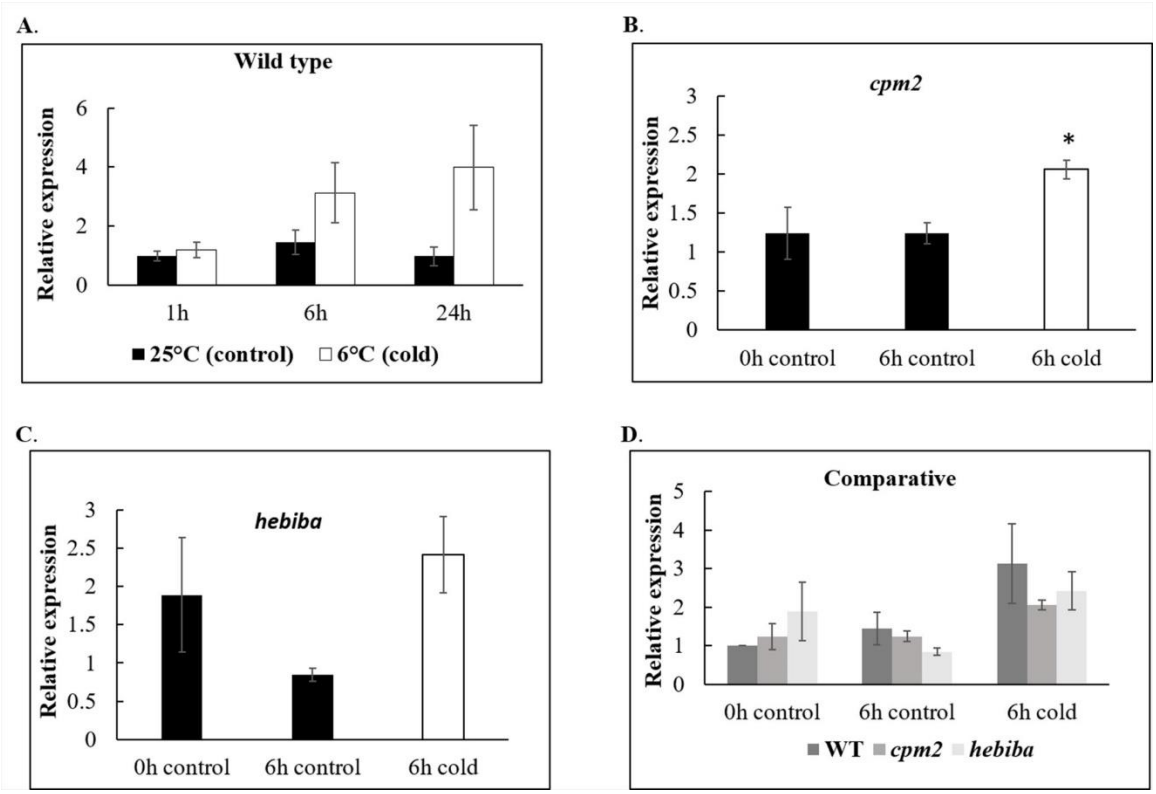

Figure 3.11: Relative gene expression analysis of JA-biosynthesis gene *OsOPR7* in the root of wild type and JA-deficient mutants *cpm2* and *hebiba* under control (25ºC) and cold (6ºC) treatment conditions considered the time duration of 0,1,6, and 24 hours for wild type whereas 0 and 6 hours for *cpm2* and *hebiba*. **(A.)** wild type root *OsOPR7*. **(B.)** *cpm2* root *OsOPR7*. **(C.)** *hebiba* root *OsOPR7*. **(D.)** root comparative graph *OsOPR7*. Data represent the average of three biological replicates with three technical replicates in each experiment. Error bars show the standard error value. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; *P < 0.05).

3.3.2. Cold stress induced the expression of JA-responsive genes *OsJAZ12* and *OsJAZ13* in wild type, but not in JA mutants *cpm2* and *hebiba*.

JA has been known to play an important role in plant response and adaptation to unfavorable conditions and various abiotic stress. It has been reported that JAZ proteins play a negative role in JA signalling. So, in our study, we considered elucidating the role of JAZ genes involved in cold stress. We have identified *OsJAZ12* and *OsJAZ13* as JA-responsive genes in wild type. In shoot of wild type, after 1 hour of cold treatment *OsJAZ12* transcripts were up-regulated. We have observed the maximum level of gene expression in 6 hours of exposure to cold stress, and its expression was a little bit weaker at 24 hours of exposure to cold (Figure 3.12 A). *Cpm2* and *hebiba* shoot showed very weak or almost no upregulation in the expression of the *OsJAZ12* gene in response to cold stress (Figure 3.12 B and C). Whereas the strong expression pattern has been observed in the root of wild type, the upregulation of gene expression was observed from 1 hour of cold exposure, increased in 6 hours exposure, and highest was observed in 24 hours of cold exposure (Figure 3.13 A). *Cpm2* and *hebiba* showed weak expression on exposure to cold stress (Figure 3.13 B and C). Therefore, we concluded that *OsJAZ12* showed a stronger expression level in the root and shoot of wild type and its expression is very weak in the shoot and root of JA biosynthesis mutants *cpm2* and *hebiba* (Figure 3.12 D and 3.13 D).

OsJAZ12 SHOOT

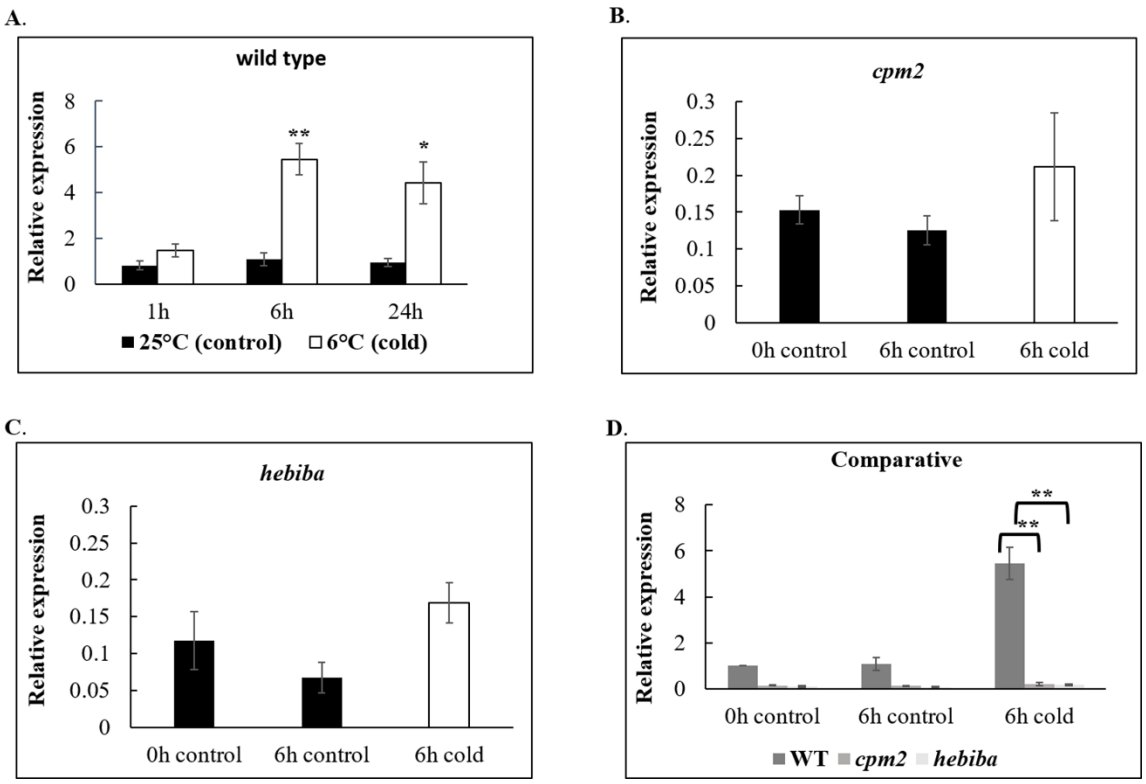

Figure 3.12: Relative gene expression analysis of JA-responsive gene *OsJAZ12* in the shoot of wild type and JA-deficient mutants *cpm2* and *hebiba* under control (25ºC) and cold (6ºC) treatment conditions considered the time duration of 0,1,6, and 24 hours for wild type whereas 0 and 6 hours for *cpm2* and *hebiba*. **(A.)** wild type shoot *OsJAZ12*. **(B.)** *cpm2* Shoot *OsJAZ12*. **(C.)** *hebiba* shoot *OsJAZ12*. **(D.)** shoot comparative graph *OsJAZ12*. Data represent the average of three biological replicates with three technical replicates in each experiment. Error bars show the standard error value. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; *P < 0.05 and **P < 0.01).

OsJAZ12 ROOT

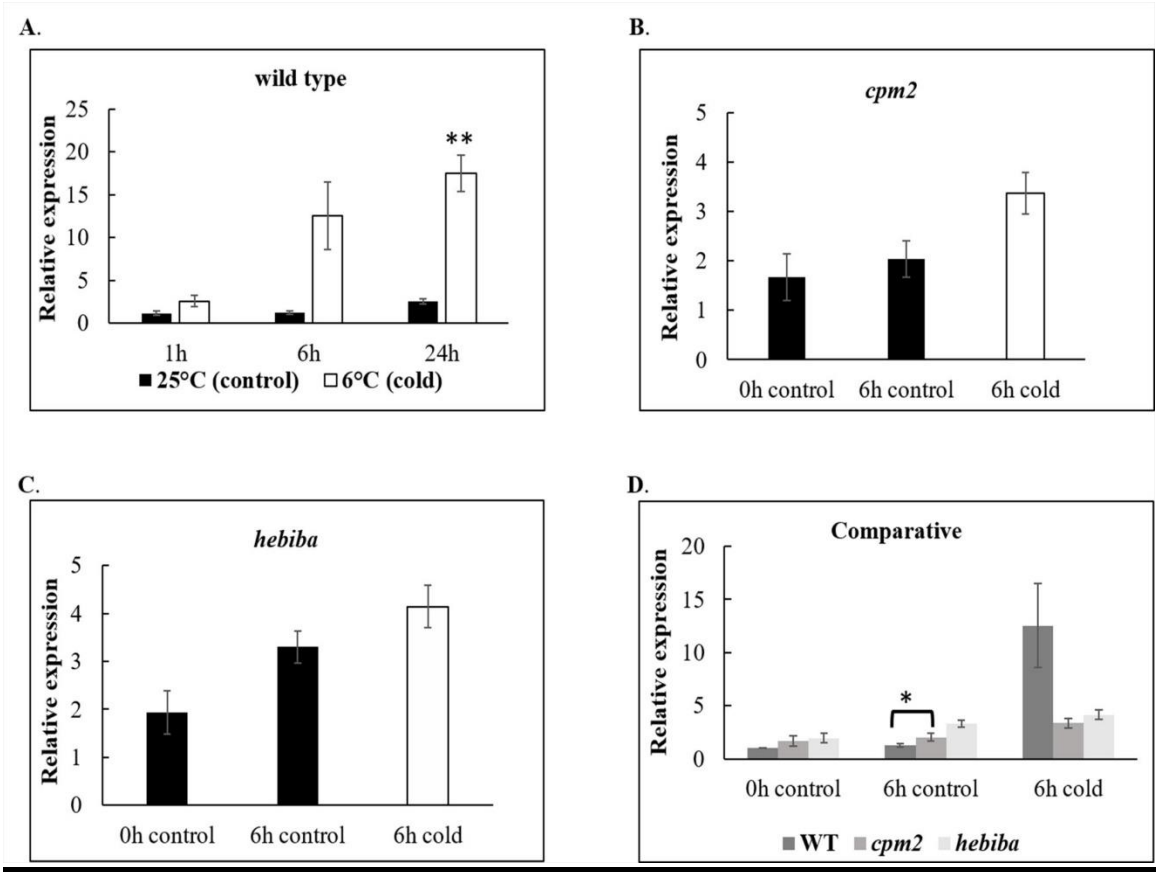

Figure 3.13: Relative gene expression analysis of JA-responsive gene *OsJAZ12* in the root of wild type and JA-deficient mutants *cpm2* and *hebiba* under control (25ºC) and cold (6ºC) treatment conditions considered the time duration of 0,1,6, and 24 hours for wild type whereas 0 and 6 hours for *cpm2* and *hebiba*. **(A.)** wild type root *OsJAZ12*. **(B.)** *cpm2* root *OsJAZ12*. **(C.)** *hebiba* root *OsJAZ12***. (D.)** root comparative graph *OsJAZ12*. Data represent the average of three biological replicates with three technical replicates in each experiment. Error bars show the standard error value. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; *P < 0.05 and **P < 0.01).

OsJAZ13 transcripts were induced after 1 hour and accumulated very strongly after 6 hours of cold stress (300-fold expression level) in shoots of the wild type. After 24 hours of cold stress, expression was going down, but still high (50-fold induction, Figure 3.14 A). The shoot of JA-deficient mutants *cpm2* and *hebiba* showed no upregulation of *OsJAZ13* genes on exposure to cold stress of 6 hours (Figure 3.14 C and D). The same but weaker expression pattern of *OsJAZ13* genes observed in the roots of wild type as compared to wild type shoots. Upregulation in the expression of the *OsJAZ13* gene with the fold induction of 60 has been observed on exposure to cold stress of 6 hours in the roots of wild type (Figure 3.15 A). The expression tends to decrease in 24 hours of exposure to cold stress with the fold induction of 40. No upregulation in the gene expression has been observed in the root of *cpm2* and *hebiba* (Figure 3.15 B and C). The result indicated that the JA responsive genes *OsJAZ12* and *OsJAZ13* are responsive to cold stress in wild type shoot and root but not in shoot and root of *cpm2* and *hebiba,* respectively, as well as *OsJAZ12* and *OsJAZ13* are only transiently expressed genes and maximally induced at 6 hours cold exposure.

OsJAZ13 **SHOOT**

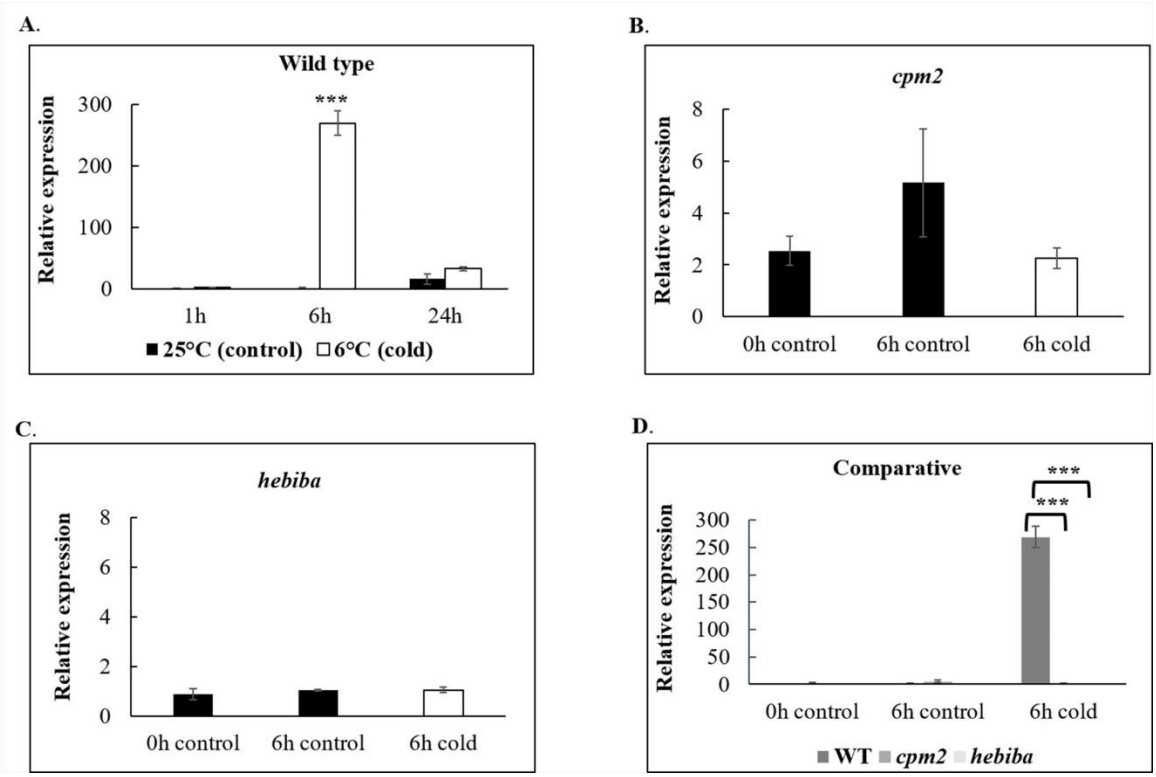

Figure 3.14: Relative gene expression analysis of JA-responsive gene *OsJAZ13* in the shoot of wild type and JA-deficient mutants *cpm2* and *hebiba* under control (25ºC) and cold (6ºC) treatment conditions considered the time duration of 0,1,6, and 24 hours for wild type whereas 0 and 6 hours for *cpm2* and *hebiba*. **(A.)** wild type shoot *OsJAZ13*. **(B.)** *cpm2* Shoot *OsJAZ13*. **(C.)** *hebiba* shoot *OsJAZ13*. **(D.)** shoot comparative graph *OsJAZ13*. Data represent the average of three biological replicates with three technical replicates in each experiment. Error bars show the standard error value. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; ***$P \leq 0.001$).

***OsJAZ13* ROOT**

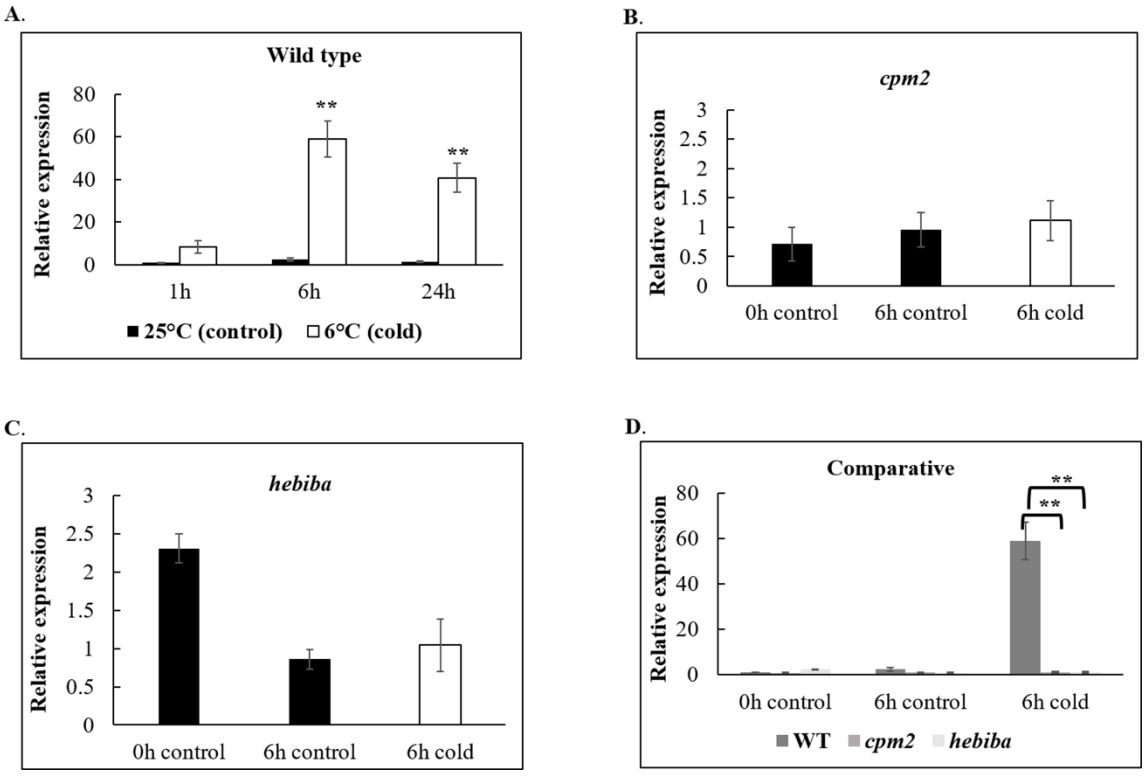

Figure 3.15: Relative gene expression analysis of JA-responsive gene *OsJAZ13* root of wild type and in JA-deficient mutants *cpm2* and *hebiba* under control (25ºC) and cold (6ºC) treatment conditions considered the time duration of 0,1,6, and 24 hours for wild type whereas 0 and 6 hours for *cpm2* and *hebiba*. **(A.)** wild type root *OsJAZ12*. **(B.)** *cpm2* root *OsJAZ13*. **(C.)** *hebiba* root *OsJAZ13*. **(D.)** root comparative graph *OsJAZ13*. Data represent the average of three biological replicates with three technical replicates in each experiment. Error bars show the standard error value. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; **P < 0.01).

3.3.3. Cold responsive gene induced strongly in shoot and root of wild type as well as the root of JA-deficient mutants *cpm2* and *hebiba*

It has been reported that *OsDREB1A* is a cold-responsive gene and has a crucial role in plant adaptation against cold stress (Diqiuyu *et al.,* 2013; Shinozaki K *et al.,* 2005; Mohapatra *et al*., 2018; Behera *et al.,* 2019). In our study, we have found that *OsDREB1A* induced on exposure to cold stress of 1 hour, and in 6 hours the strong upregulation has been recorded with the fold-induction of 400 that tended to decrease in 24 hours with the fold-induction of 100 in the shoot of wild type (Figure 3.16 A). *Cpm2* showed very weak upregulation of *OsDREB1A* with the fold induction of 9 and *hebiba* shoot showed no upregulation in transcripts level in response to cold stress of 6 hours (Figure 3.16 B and C). This result indicated the *OsDREB1A* is a short-lived gene and very strongly induced in wild type shoots but not in the shoots of *hebiba* and *cpm2* which lead us to the conclusion the expression of *OsDREB1A* in shoots is JA-dependent.

However, in roots, no such pattern has been observed. *OsDREB1A* was induced with the same amplitude in the roots of wild type and *cpm2* in response to cold stress of 6 hours with the fold induction of 80, whereas *hebiba* showed the highest upregulation in the gene expression with approx. 500-fold induction (Figure 3.17 A, B, and C). After exposure to cold stress for 24 hours, we observed a decrease in the amplitude of upregulation in the gene as compared to 6 hours in wild type root (Figure 3.17 A). So, we concluded that *OsDREB1A* acts differently in different tissues, i.e. roots and shoots. In the root, the expression of *OsDREB1A* is not dependent on

JA as well as the expression was not as strong as we have found in the shoot of wild type. *OsDREB1A* was found to be transiently induced.

***OsDREB1A* SHOOT**

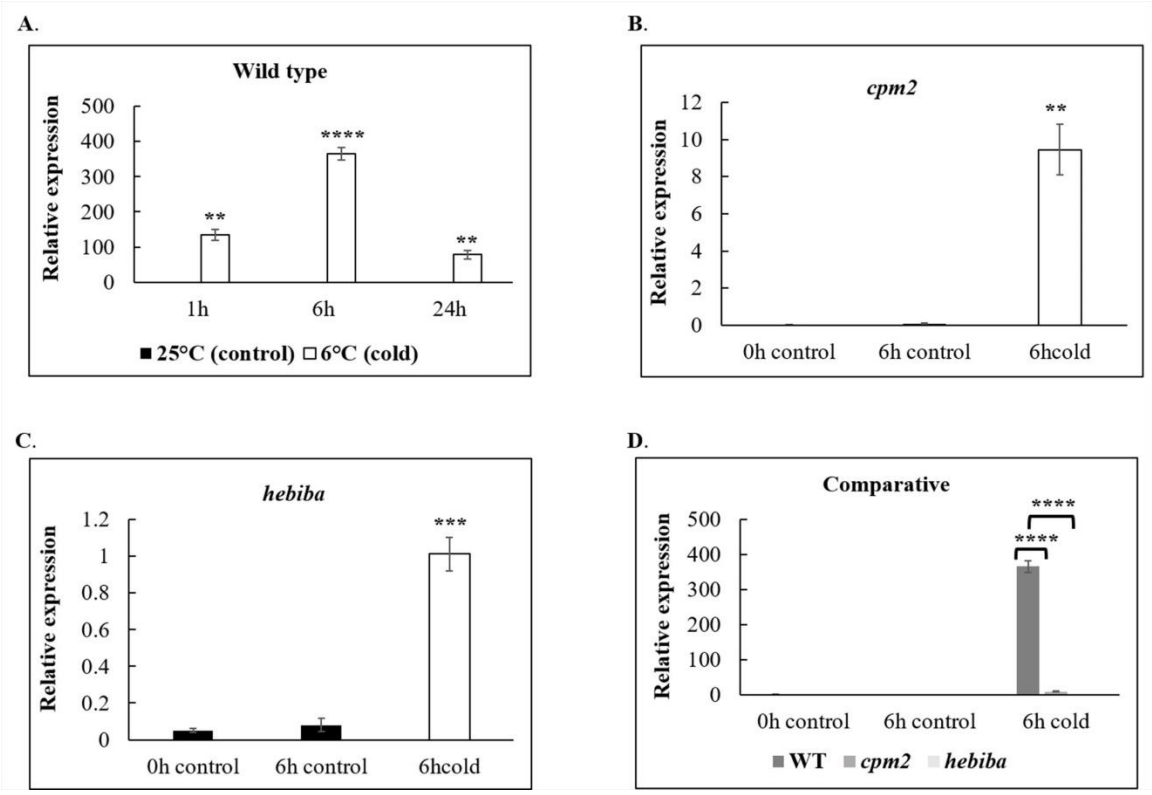

Figure 3.16: Relative gene expression analysis of cold-responsive gene *OsDREB1A* in the shoot of wild type and JA-deficient mutants *cpm2* and *hebiba* under control (25ºC) and cold (6ºC) treatment conditions considered the time duration of 0,1,6, and 24 hours for wild type whereas 0 and 6 hours for *cpm2* and *hebiba*. **(A.)** wild type shoot *OsDREB1A.* **(B.)** *cpm2* Shoot *OsDREB1A*. **(C.)** *hebiba* shoot *OsDREB1A.* **(D.)** shoot comparative graph *OsDREB1A*. Data represent the average of three biological replicates with three technical replicates in each experiment. Error bars show the standard

error value. The asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; **P < 0.01, ***P ≤ 0.001 and****P ≤0.0001).

***OsDREB1A* ROOT**

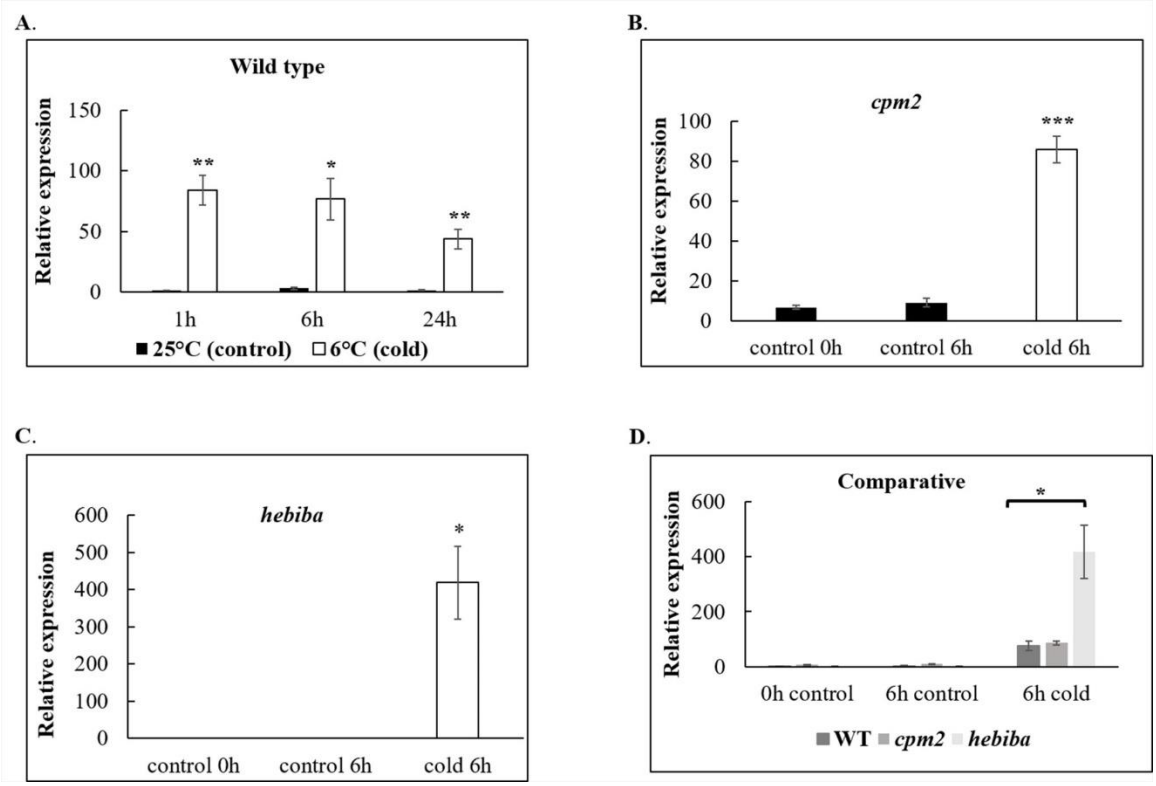

Figure 3.17: Relative gene expression analysis of cold-responsive gene *OsDREB1A* in the root of wild type and JA-deficient mutants *cpm2* and *hebiba* under control (25ºC) and cold (6ºC) treatment conditions considered the time duration of 0,1,6, and 24 hours for wild type whereas 0 and 6 hours for *cpm2* and *hebiba*. **(A.)** wild type root *OsDREB1A.* **(B.)** *cpm2* root *OsDREB1A.* **(C.)** *hebiba* root *OsDREB1A.* **(D.)** root comparative graph *OsDREB1A.* Data represent the average of three biological replicates with three technical replicates in each experiment. Error bars show the standard error value. The

asterisk indicates statistically significant differences between the control and cold treatment (Student's t-test; *P <0.05, **P < 0.01 and ***P ≤ 0.001).

3.3.4. To check the pathway involved activation of cold-responsive genes through activation of phospholipase instead of JA

Phospholipids are considered as water and fat-soluble compounds present in the lipid bilayer structure of cell membranes in plants. The drop in temperature in plants is first sensed by the cell membrane, and induce changes in Ca^{2+} (increases the cytosolic Ca^{2+} ion concentration) (Ruelland *et al*. 2002). The PLD (Phospholipase) enzyme gets activated and cleaves the phospholipase into Phosphatic acid (PA) which act as a secondary messenger and triggers the cold signalling pathways by activating DREB1A/CBF3 which binds to CRT/DRE cis-element in the promoter regions of COR genes and thus activates the transcription of COR (Stockinger *et al.* 1997; Liu *et al.*1998; Thomashow 1999; Ruelland *et al.*, 2005; Maruyama *et al.*, 2012; Furumoto *et al.*, 2013). Hence in our study, we used 0.5% (v/v) n-butanol in addition to cold stress to treat the roots of wild type to block the activity of PA a secondary messenger.

In the roots of wild type, we found the transcriptional expression of the JA-responsive genes *OsJAZ12* and *OsJAZ13* and JA-biosynthesis gene *OsAOS2* were repressed in n-butanol treated roots (Figure 3.18 A, B, and C.). It indicated that along with JA, PA is also necessary to regulate the expression of JA-responsive genes and JA-biosynthesis genes.

We found from our experiment the expression pattern of *OsDREB1A* a cold-responsive gene was partially inhibited in response to n-butanol treatment in

the root of wild type (Figure 3.18 D). The result indicated that for activation of *OsDREB1A* there may be a third factor other than PA and JA needed.

Wild type treated with n-butanol 0.5% (v/v)

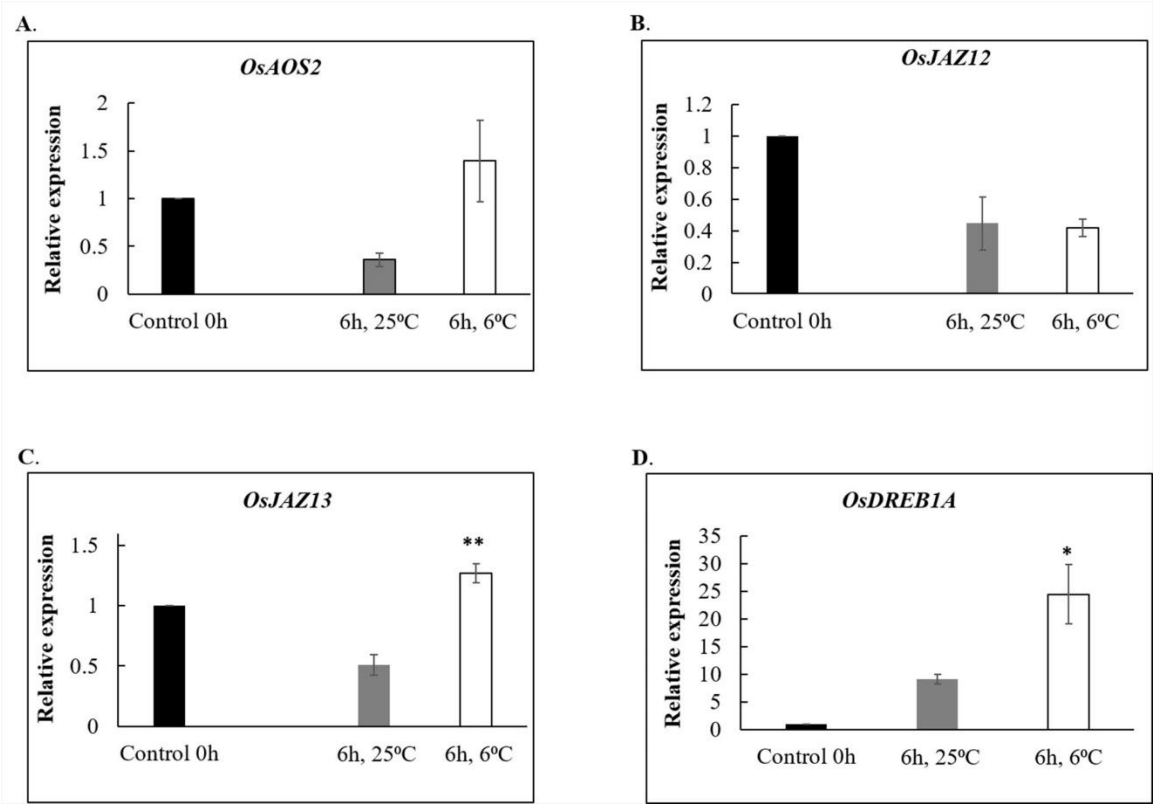

Figure 3.18: Relative gene expression analysis of JA-biosynthesis gene OsAOS2, JA-responsive genes OsJAZ12 and OsJAZ13, and cold-responsive gene OsDREB1A in wild type root treated with n-butanol 0.5% (v/v) under control (25ºC) and cold (6ºC) treatment conditions considered the time duration of 0 and 6 hours **(A.)** Root *OsAOS2*.**(B.)** Root *OsJAZ12*. **(C.)** Root *OsJAZ13*. **(D.)** Root *OsDREB1A*. Data represent the average of three biological replicates with three technical replicates in each experiment. Error bars show the standard error value. The asterisk indicates statistically

significant differences between the control and cold treatment (Student's t-test; *P < 0.05 and **P < 0.01).

3.4. Summary of the result

In this study, the obtained data were used to study the cold-triggered relative changes evolved as an adaptation process in the wild type rice cultivar Nihonmasari compared to JA-deficient mutants *cpm2* and *hebiba*. The following observations were reported:

1. Morphological analysis revealed that the damaged symptoms triggered by cold stress such as weak culm, late leaf formation, less dry weight content, and less relative water content were more pronounced in JA-deficient mutants than the wild type.
2. Biochemical analysis showed that the JA-deficient mutants were affected more than the wild type under cold stress, as mutants accumulated more MDA in the shoots and roots and less chlorophyll in the leaves compared to the wild type.
3. Transcriptional analysis revealed that the JA-biosynthesis gene *OsAOS2* was responsive to the cold stress in the shoots and roots of wild type as well as mutants, indicating that the expression of *OsAOS2* is independent of JA.
4. The JA-signalling genes *OsJAZ12* and *OsJAZ13* were responsive to cold stress in shoots and roots of wild type but not in the shoots and roots of mutants, indicating that the expression of *OsJAZ12* and *OsJAZ13* is dependent on JA.
5. The transcriptional expression of cold-responsive gene *OsDREB1A* was induced on exposure to cold stress in shoots and roots of wild type as well

as mutants indicating organ-specific expression of *OsDREB1A* i.e. JA-dependent and -independent expression in shoots and roots, respectively.

6. Transcriptional expression of *OsDREB1A* in n-butanol treated roots of the wild type demonstrated the involvement of unknown factor ‘X’ other than JA and PA to regulate the expression of *OsDREB1A* in roots.
7. Phytohormonal measurement in shoots of wild type supports the result of transcriptional analysis as endogenous accumulation of OPDA, JA, and JA-Ile has been observed under cold stress.
8. In the root of wild type in contrast to transcriptional analysis no accumulation of JA and JA-Ile, on exposure to cold stress has been observed whereas OPDA was slightly accumulated.

4. DISCUSSIONS

4.1. The contrasting role of JA under abiotic stresses

It has been reported that Jasmonic acid is a lipid-derived phytohormone which is widely studied as a regulator of plant growth and development and also known to induce defence mechanisms in plants under biotic and abiotic stress conditions. There are contradictory reports on the role of JA under abiotic stress We already know that jasmonate function in abiotic stress tolerance depends on the stressors. Previous study from our own group show that jasmonate can be a positive regulator (Osmotic stress, Tang *et al.,* 2020) or negative regulator (Salinity stress, Hazman *et al.,* 2015; Peethambaran *et al.,* 2018) for example, in rice under salinity stress, it has been reported, that reduction or inactivation of jasmonate results in better adaptation under salt stress as JA-deficient mutants of rice *cpm2* and *hebiba* (Hazman *et al.,* 2015) and CYP94C2b (gene encoding an enzyme required for metabolizing jasmonic acid) overexpressing transgenic plant (Kitoka *et al.,* 2011; Kurotani *et al.,* 2015) showed increased salt tolerance compared to their wild type. Therefore, it is difficult to make a positive correlation between JA and abiotic stresses in general, but the specific form of abiotic stress (i.e. the stressor) has to be considered. However, the role of JA has been studied intensively under cold stress, some studies revealed JA plays a positive role under cold stress as defence genes like CBF are controlled by JA signalling in *Arabidopsis* (Hu *et al.,* 2013). There are several reports which revealed that an exogenous application of JA helps to cope up with multi-stress factors in plants (Rosahl and Feussner 2005; Ahmadi *et al.,* 2018; Tayyab *et al.,* 2020). Whereas, the formation of secondary messengers such as Phosphatidic acid also linked with

activation of cold-responsive genes. Therefore, we employed two AOC mutants *cpm2* (AOC specific mutant) and *hebiba* (additional mutations also present) as JA-deficient genotypes and Nihonmasari as a wild type to study the role of JA under cold tolerance in rice and in the second part of study, the secondary messenger PA (Phosphatidic acid) was targeted by treating the wild type root with n-butanol to understand if PA-dependent signalling pathway involved in cold-tolerance mechanism.

4.2. Morphological, physiological and biochemical changes in wild type and jasmonic acid mutants on exposure to cold stress.

Cold stress triggers plant responses such as changes in growth and development, yield, changes in cellular metabolism and biochemical process. Understanding the cold-triggered changes in biochemical and molecular level is important to get a deeper insight of plant resistance response under cold-stress conditions. So, in this study some aspects of cold-induced morphological, physiological, biochemical changes have been discussed.

4.2.1. JA alleviates the effect of cold stress on plant growth

We observed the response of JA-deficient mutant and wild type plants to cold stress imposed in a hydroponic system at 6°C, as cold stress adversely affects the growth and development of rice plants. Leaf development in rice is governed by undergoing cell division in response to changing environmental conditions. Chilling stress causes stunted and weak seedling growth as well as alters the shoot development by prolonging cell division followed by the production of a lesser number of cells. It slows down the rate of leaf initiation period (Warrington and Kanemasu, 1983; Lukatkin *et al.*, 2012; Jouyban *et*

al., 2013 and Hussain *et al.*, 2018). This results into a reduction in the formation of new leaves in the period of cold stress. In our study (Figure 3.1) under cold stress conditions, wild type showed fully expanded tertiary leaves whereas two JA-deficient mutants *cpm2* and *hebiba* showed complete absence of tertiary leaves. Wild type displayed strong and healthy (green) culm, whereas the culms of *cpm2* and *hebiba* were very weak and yellow which lead to instability of the whole plant. In addition, we found that *cpm2* as well as *hebiba* were affected more strongly when exposed to cold stress than the wild type. Hence it suggested that JA ameliorates symptoms caused by cold stress in rice.

4.2.2. JA mitigates the effect of cold stress on physiological level by improving growth performance

Various physiological parameters were evaluated to quantify the impact of cold stress on the wild type and mutants. Relative water content (RWC) is considered as an important attribute to measure plant-water relationships as well as parameter for determining cellular water deficit conditions of plants as a physiological consequence due to stress. It has been reported, under cold stress conditions there is a reduction in leaf water potential as a result of osmotic adjustment (Aroca *et al.*, 2003; Farooq *et al.*, 2009a; and Sales *et al.*, 2013) We found RWC declined under cold stress condition in all the genotypes. Wild type was affected least compared to the JA-deficient mutants as cold treated leaves of wild type showed only 10% reduction in RWC compared to control whereas in *cpm2* and *hebiba* it was 12.9 and 12.4%, respectively (Figure 3.2).

Tolerant genotypes showed better shoot traits such as green stem, less reduction in the shoot length, and less difference in biomass accumulation. In

our study *cpm2* and *hebiba* were affected more under cold stress in comparison with wild type as the slower growth rate has been observed in the form of significantly decreased root and shoot length, more differences in the shoot biomass accumulation and absence of tertiary leaves. Chilling stress alters the root growth by decreasing the length of root and biomass accumulation (Cutforth *et al.,* 1986), we found there was a difference in the root length as well as in biomass accumulation in JA mutants and wild type under control and cold treated conditions. Reduction in the dry weight occurred to manage and drive photosynthesis as under stress condition roots may not able to take up more water, thus plants maintain a balance between photosynthesis and root growth. The mutant *hebiba* was affected more and we found there was more difference in the dry weight and root length in control and cold treated seedling of *hebiba* than wild type and *cpm2*, this lead us to the conclusion that JA not alone but other factors also contribute to change in root response on exposure to cold stress.

4.2.3 JA alleviates the effect of cold stress on chlorophyll content and MDA

Chlorophyll is an extremely important component for photosynthesis as it is crucial in the absorption of light energy and light energy transformation and to evaluate the photosynthetic capacity of plants. Previous studies have revealed that various biotic and abiotic stress result into a reduction in chlorophyll content. Cold stress is one of the major factors causing inhibition in the biosynthesis of chlorophyll, interference with the utilization of light energy and it can therefore influence plant photosynthesis (Xu *et al.,* 2000; Glaszmann *et al.,* 1990 and Wu *et al.,* 1997). We examined the content of

chlorophyll in the control and cold treated seedlings of wild type and JA-deficient mutants. Compared with the control, the chlorophyll content in seedling leaves under low temperature was lower than that in control temperature probably due to cold stress suppresses the biosynthesis of chlorophyll by inhibiting the activities of an enzyme involved in the biosynthesis of chlorophyll, however the mechanism by which chlorophyll synthesis is inhibited under cold stress is still unknown (Zhao *et al.,* 2020). Among all the genotypes, the wild type was affected least in the reduction of chlorophyll content by 10%, whereas in *cpm2* and *hebiba* it was 18 and 23 %, respectively, in cold treated seedlings compared to their control (Figure 3.4). This led us to the conclusion, cold stress affects the photosynthetic pigments and JA mitigates the effect of cold stress as we found wild type was least affected by cold stress. But in case of *cpm2* and *hebiba,* we found *hebiba* has more reduction in the chlorophyll contents, which might be caused by the lack of additional genes in *hebiba*.

Cold stress often causes injuries to the cell membrane and malondialdehyde (MDA) is an important indicator of membrane system injuries (Liu *et al.*, 2013; Bhattacharjee 2013; Awashthi *et al.*, 2015; Hsu and Hsu 2019; Zhao et al., 2020). In our study, to examine oxidative damage and lipid peroxidation, we measured MDA content in the root and shoot of cold and control-treated seedlings of wild type, *cpm2* and *hebiba.* We found MDA content increased on exposure to cold stress, in both, roots and shoots of wild type, *cpm2* and *hebiba*. Among the three genotypes, wild type has accumulated less MDA content in the roots and shoots of cold treated seedling compared to control. Whereas, *cpm2* and *hebiba* has more accumulation of MDA in the roots and shoots of cold treated seedling compared to control. Therefore, we concluded, chilling stress induces the lipid peroxidation as MDA content increased on

exposure to cold stress and thus, causes damage to the integrity of cellular membrane, but JA reduces the effect of cold stress on a biochemical level as wild type was least affected compared to *cpm2* and *hebiba.*

4.3. Cold stress induced JA-independent activation of *OsAOS2*, JA-dependent activation of *OsJAZ12*, OsJAZ13 and differential accumulation of OPDA, JA and JA-Ile in shoots and roots of wild type

In the second part of the study, we have checked the expression of JA-biosynthesis, JA-responsive, cold-responsive gene in shoots and roots of wild type and JA-deficient mutants under cold stress. We also determined phytohormones such as OPDA, JA, JA-Ile, ABA and SA level in roots and shoots of wild type. The reported genes involved in the biosynthesis of JA in rice genome contain *OsDAD1, OsLOX2, OsAOC, OsAOS1, OsAOS2, OsOPR1, and OsOPR7* and these genes are induced by cold and drought stress (Du *et al.,* 2013). Microarray analysis revealed the upregulation of the JA-biosynthesis gene under cold stress (Sharma *et al.,* 2016). As many studies suggested the positive role of Jasmonate in cold tolerance in rice, in order to analyse the involvement of jasmonate's role in cold tolerance mechanisms, we tested the transcriptional level of the jasmonate biosynthesis genes *OsAOS2* and surprisingly we found that the *OsAOS2* was not only upregulated in shoots and roots of wild type but also in the shoots and roots of JA-deficient mutants on exposure to cold stress (Figure 3.8 and Figure 3.9). As JA-deficient mutants have impaired *OsAOC* gene and *OsAOS2* is upstream of *OsAOC,* therefore, the upregulation in the expression of *OsAOS2* is induced by low temperatures independent of JA. *OsOPR7* was upregulated in the

shoots of wild type but not in the shoots of JA-deficient mutants (Figure 3.10). However, in the roots of wild type, *cpm2* and *hebiba* we found the upregulation in the transcript level (Figure 3.11). The JA-responsive *OsJAZ12* and *OsJAZ13* genes were responsive to cold stress in shoot and root of wild type but not in shoots and roots of mutants, which provides evidence that the expression of *OsJAZ12* and *OsJAZ13* genes are JA-dependent. The cold-responsive *OsDREB1A* gene was upregulated in shoots and roots of wild type and surprisingly, also in the root of *cpm2* and *hebiba* providing evidence for organ-specific cold regulation of *OsDREB1A* i.e. JA-dependent in shoots and JA-independent in roots. From the phytohormonal measurement, on exposure to cold stress, JA, JA-Ile, and its precursor OPDA were strongly induced and significantly accumulated in the shoots of the wild type which supports our transcriptional analysis data but no accumulation of ABA and SA has been observed. In the roots of wild type, no endogenous accumulation of JA, JA-Ile and SA were observed on exposure to cold stress of 1, 6, and 24 hours, but increase in the endogenous level of OPDA was found. However, ABA is slightly accumulated. So based on our result there might be two possibilities i.e. we did not hit the right time-point for hormonal analysis to find a JA-Ile peak or not JA-Ile but another jasmonate is active as it has been reported that depending on the modification in the pentenyl chain, carboxyl-acid group, and pentanone ring, JA can be metabolized into cis-jasmone by decarboxylation of JA, JA-glucosyl ester, 12-O-gulcosyl-JA, JA-Ile and other forms (Koch *et al.,* 1997; Li *et al.,* 2018), among which JA-Ile, free JA, cis-jasmone, and MeJA are considered as a major form of bioactive JA (Wasternack *et al.,* 2013) . Based on differential expression of *OsDREB1A* in shoot and root, we proposed a model i.e. JA-dependent and JA-independent induced expression of *OsDREB1A* in shoot and root respectively.

4.3.1. JA-dependent pathway in shoot: JA regulates the ICE-CBF/DREB signalling pathway and induces cold tolerance

Rice genome includes 15 *OsJAZ* genes (Ye *et al.,* 2009). JAZ genes are known to involve in JA-signalling pathways (Du *et al.,* 2013). Studies have revealed the transcription factors downstream to JAZ are involved in regulating cold responses as cold stress activates the expression of CBF family TF, which further activates downstream genes and involved in regulating of cold responses. The role of JAZ in regulating the cold tolerance pathway has been investigated, as JAZ act as a repressor for ICE under normal conditions and suppresses the ICE-CBF/DREB signalling pathway. *OsDREB1A* gene is identified as a cold-responsive gene in rice (Dubouzet *et al.,* 2003; Ito *et al.,* 2006). The overexpression of *OsDREB1A* in transgenic Arabidopsis enhanced cold tolerance (Liu *et al.,* 1998; Kasuga *et al.,* 1999; Gilmour *et al.,* 2000; Dubouzet *et al.,* 2003; Maruyama *et al.,* 2004; Oh *et al.,*2005). There are many genes present downstream of DREB1A, because their activation under cold stress needs activation of DREB1A and DREB1A acts as a transcription factor for COR (cold-responsive gene) (Maruyama *et al.,* 2004). The expression of DREB1A is induced by upstream TF ICE1 upon MeJA treatment under cold stress. The transgenic overexpressing DREB1A rice plants showed tolerance towards cold stress and contain a higher amount of osmoprotectants such as soluble sugar and proline. In order to get the correlation between JAZ protein and ICE, it has been found that the transgenic plants which have overexpressed JAZ proteins are more sensitive towards cold stress because the overexpressed JAZ repressed the transcription activity of CBF/DREB1A (Hu *et al.,* 2013). JAZ proteins act as a repressor, controlling the JA-signalling

pathway and the cold stress tolerance as, under normal conditions, JAZ repressors target ICE proteins and repress the transcription activity ICE (Hu *et al.*, 2013). In our study, we found *OSJAZ12* and *OsJAZ13* were responsive towards cold stress and induced upon cold exposure in shoot and root of wild type but not in the shoot and root of JA-deficient mutants. In the JA-dependent cold-signalling pathways, on exposure to cold stress, receptor COI1 perceives jasmonates and they cause the degradation of JAZ protein as a result of which ICE becomes free from the repression of JAZ repressor. ICE act as a transcription factor and induces the expression of DREB1A which induces the expression of COR and thus facilitates cold tolerance in plants **(Figure 4.2)** (Chinnusammy *et al.*, 2003; Hu *et al.*, 2013).

4.3.2. JA-independent: Involvement of secondary messenger PA to induce the expression of *OsDREB1A* or PA-dependent cold signalling pathway worked in root

The calcium influx takes place as an early event during cold stress. From our result, we hypothesize that there might be a possible link between calcium-dependent MAPK cascade and cold signalling. As it has been already revealed that the on exposure to cold stress, there is an increase in the concentration of cytosolic $Ca^{2+,}$ activation of Phospholipase D (PLD), formation of secondary messenger phosphatidic acid (PA), activation of MAP Kinase pathway and induction of stress-responsive genes (Knight *et al.*, 1993; Örvar *et al.*, 2000; Tuteja and Mahajan 2007). On exposure to cold stress, calcium influx into the cells activates the lipid-signalling enzyme PLD. The cold stress induced biochemical changes first sensed by phosphatidylcholine, a class of phospholipid and the major component of lipid bilayer structure as it has

hydrophilic (polar) head and hydrophobic (non-polar) tail. Phosphatidylcholine has been found to be involved into early cold signalling. PLD hydrolyses phosphatidylcholine and generates secondary messenger phosphatidic acid and water-soluble choline. Phosphaditic acid (PA) (Ruellend *et al.*, 2002; Vergnolle *et al.*, 2005), the secondary messenger signature induced by cold is decoded by mitogen-activated protein kinase cascade (MAP cascade) which gets activated and involved in cold signalling and cold tolerance. The PLD pathways are present upstream of CBF and induces the expression of CBF/DREB1 in *Arabidopsis* (Teige *et al.*, 2004). In order to get insight whether PA-dependent signalling events influence jasmonate signalling and if the blocking of PA under cold stress can inhibit the expression of *OsDREB1A*, the roots of wild type were treated with 0.5% (v/v) n-butanol to manipulate the activity of lipid-signalling PLD enzyme. In the presence of n-butanol, PLD not only transfer the phosphatidyl group of phosphatidylcholines to water for the formation of active secondary messenger PA but also to n-butanol to form an inactive phosphatidylbutanol as there is competition between n-butanol and water as a nucleophile to accept phosphatidyl group. Hence the activity of PA is blocked **(Figure 4.1.)**. From our study, the expression of *OsDREB1A* was found to be partially inhibited in the n-butanol treated roots of wild type on exposure to cold stress, which provides an evidence that *OsDREB1A* was not completely blocked by PA and there is unknown factor 'X' other than JA and PA. Hence an intersection of JA and PA-cold signalling pathway to regulate the activation of *OsDREB1A* on exposure to cold stress must exist in the root **(Figure 4.2).** In contrast, the transcriptional expression of *OsAOS2*, *OsJAZ12* and *OsJAZ13* was completely blocked in the n-butanol treated roots of wild type suggesting the important role of PA on the JA-signalling pathway in roots.

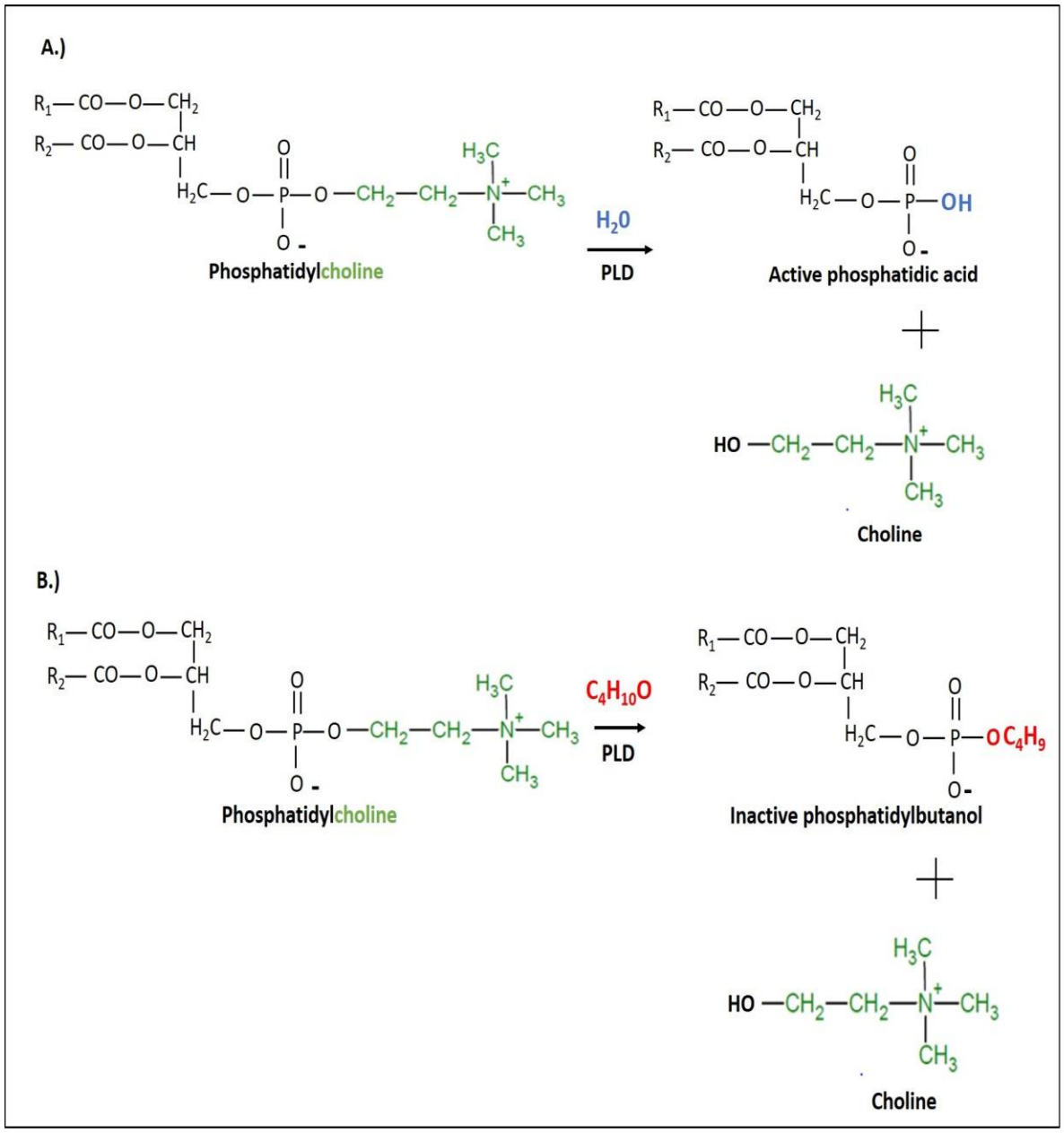

Figure 4.1: Hydrolysis of phosphatidylcholine catalysed by PLD in H_2O and n-butanol. **A.)** In the presence of H_2O, PLD transfers the phosphatidyl moiety of phosphatidylcholine to water and forms the active secondary messenger PA. **B.)** In the presence of n-butanol, PLD transfers the phosphatidyl moiety

of phosphatidylcholine to n-butanol and form an inactive phosphatidylcholine (Figure modified from O'Reilly *et al.*, 2015).

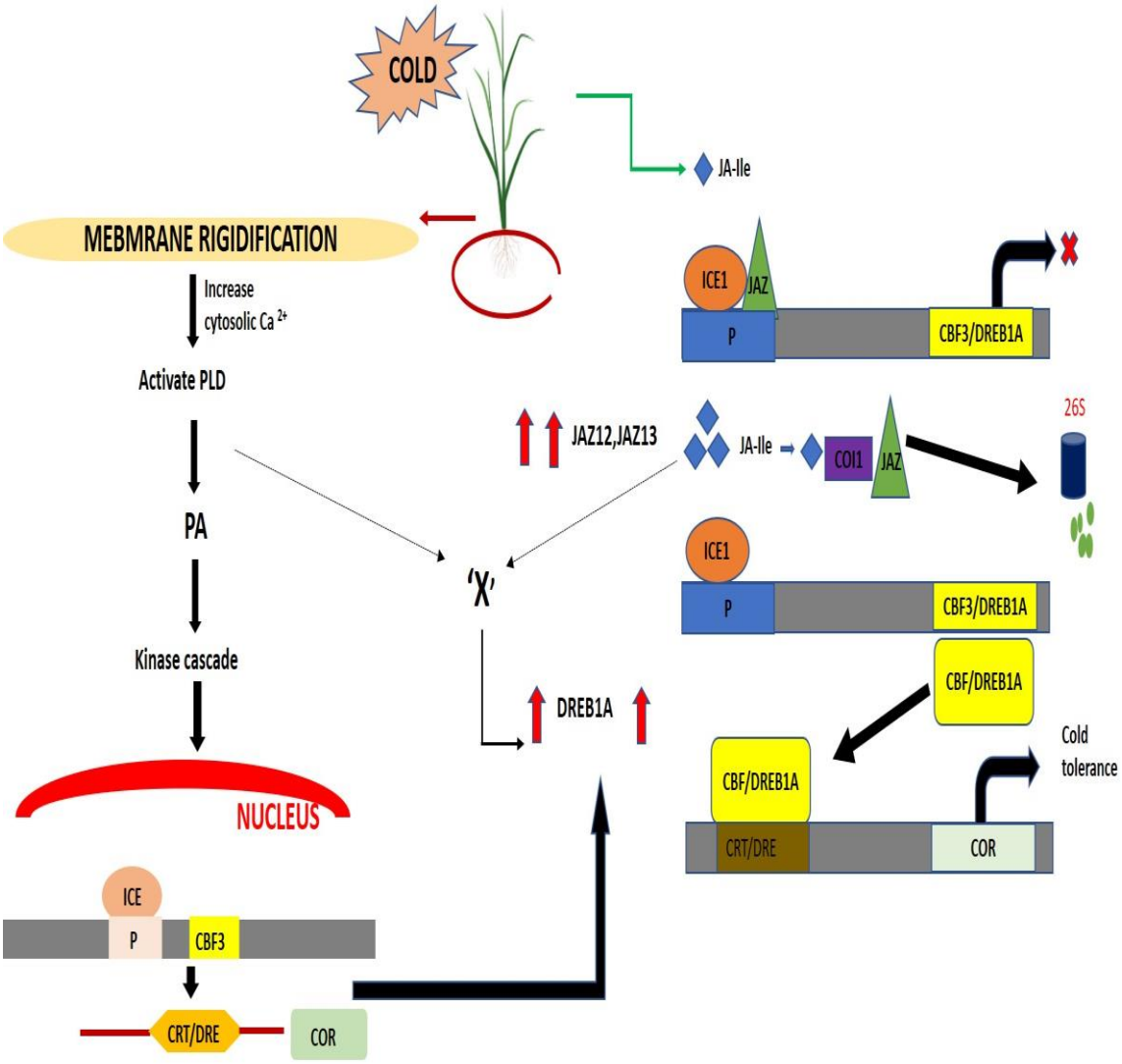

Figure 4.2: A proposed model for JA-dependent and JA-independent cold-signalling pathway operating to regulate the expression of *OsDREB1A* in shoots and roots respectively. **(A.)** Model for cold signalling pathway operating in shoots dependent on jasmonic acid. Under normal growth conditions, JAZ repressor binds with ICE transcription factor and represses

the activation of ICE. The physical interaction of JAZ and ICE attenuates the activation of cold-responsive downstream genes. On exposure to cold stress, the endogenous jasmonates levels increased and COI1 receptor catalyses the degradation of JAZ repressor as a result of which ICE will be free from repression and activates CBF3/DREB1A. Subsequently, cold-responsive genes are induced. **(B.)** Model for cold signalling pathway operating in roots independent of jasmonic acid. On exposure to cold stress, membrane rigidification takes place and increases in the cytosolic Ca^{2+}. Ca^{2+} acts as a secondary messenger and activates the enzyme Phospholipase D (PLD). PLD cleaves phospholipase into phosphatidic acid which acts as a secondary messenger and induces the MAPK cascade that activates the induction of CBF3/DREB1A gene. The unknown factor 'X' represents the third factor, other than JA and PA, intersects the JA-dependent and PA-dependent signalling pathway and induce the activation of the *DREB1A* gene on exposure to cold stress.

5. CONCLUSION

In this thesis, I am giving a deeper insight into the role of JA in the cold response of rice using JA-deficient mutants. Transcriptional analysis of JA-biosynthesis genes, JA-responsive genes, and cold-responsive genes were performed. Surprisingly we observed the transcriptional activation of the *OsDREB1A* gene in shoot and root of wild type as well as in the root of mutants, suggesting the expression of the *OsDREB1A* gene is organ-specific and we concluded the expression of *OsDREB1A* in the shoot is dependent of JA whereas in the root it was independent of JA. Furthermore, to understand if the expression of the key regulator of cold response *OsDREB1A* in roots is regulated via PA-dependent cold-signalling pathway the major lipid-signalling enzyme was manipulated by the application of 0.5% (v/v) n-butanol to block the formation of PA. We observed partial inhibition in the expression of *OsDREB1A* thus we concluded that there is an unknown factor 'X' intersecting the JA-dependent and PA-dependent cold-signalling pathways and regulates the expression of *OsDREB1A* under cold stress in roots. Our result suggests that *OsDREB1A* is a cold-responsive gene and induces cold tolerance mechanism but for more clarification, future work must be extended to measure the transcriptional activity of other cold-responsive genes. Secondly, in our study, we have found the transcriptional upregulation of JA-responsive genes in the root of wild type but no accumulation of JA and JA-Ile has been observed. Therefore, we need to consider the early time point to obtain the maximum peak of JA or to the measurement of other bioactive forms of JA is required. Conventional breeding is a time taking approach to develop resistant crops, genetic approaches or marker-assisted approaches should be considered and, in our study, we have identified major TFs involved to induce the cold-responsive gene and to switch on cold tolerance

mechanisms. So, by modifying various upstream and downstream signalling genes, a resistant crop that will be able to adapt adverse cold stress can be developed. However, only a limited number of studies have been done on hormonal crosstalk and the role of different signalling pathways on enhancing cold tolerance. Unravelling these complex networks, and using modern methods of breeding and genetic engineering, will allow achieving important progress towards cold-tolerant crops.

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