Stress defense in rice: how jasmonates enhance resistance to osmotic stress

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Karlsruhe, im Oktober 2018

Gangliang Tang

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I feel that time has passed so quickly. When I typed my final word on my computer, I had to acknowledge that my doctoral career was coming to an end, as well as my time as a student. I have been studying at KIT for almost three years, and it seems to have all happened only yesterday. My first time abroad was my trip to Germany. On the first day, I was fortunate to meet Peter. I felt curious and worried about the unfamiliar environment in Germany, and feared that I did not leave a good first impression on my supervisor. So much has happened in the past three years. I have met many good people. I have been happy, sad, laughed, cried, shared good times with friends, and reflected in solitude. I have also grown a lot. I have become inner-motivated and self-directed. I have met so many wonderful people in the past three years. We have studied, lived, and played together. Today, I need to thank all of these wonderful people.

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Gangliang Tang

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Abbreviations

13-LOX: 13-LIPOXYGENASE

13-HPOT: 13-hydroperoxyoctadecatrienoic acid

12,13-EOT: 12,13(S)-epoxyoctadecatrienoic acid

α-LeA: α-linolenic acid

ABA: abscisic acid

ACC: 1-aminocyclopropane-1-carboxylic acid

ACX: ACYL-CoA OXIDASE

ADC: Arginine decarboxylase

ANOVA: one-way analysis of variance

AOC: allene oxide cyclase

AOS: allene oxide synthase

ASP: Aspartic acid

AtHK1: Arabidopsis thaliana histidine kinase-1

BR: brassinolide

bZIP: basic leucine zipper transcription factor

CO_2: carbon dioxide

COR: cold-responsive protein

COI1: CORONATINE INSENSITIVE 1

cpm2: coleoptile photomorphogenesis 2

DM: dry mass

DRE: drought response element

DREB: dehydration response element binding

ESTs: Expressed Sequence Tags

FAD: FATTY ACID DESATURASE

FM: fresh mass

GA: gibberellin

GC-MS: Gas chromatography-mass spectrometry

GH3: GRETCHEN HAGEN3

Gs: Stomatal conductance

GSA: glutamate-semialdehyde

HSP: heat shock protein

JA: jasmonic acid

JMT: Jasmonic acid carboxyl methyltransferase

JA-Ile: jasmonoyl-isoleucine

JAZ1: jasmonate-zim-domain protein

JAR1: JASMONATE RESISTANT 1

KAT: L-3-KETOACYL-CoA THIOLASE

LEA: late embryo abundant

MDA: Malondialdehyde

MFP: multifunctional protein

MeJA: methyl jasmonate

NBT: nitroblue tetrazolium

NO: nitric oxide

$O_2^{\cdot-}$: superoxide anion

OAT: ornithine-delta-aminotransferase

OPDA: 12-oxophytodienoic acid

OPR: OPDA REDUCTASE

PAL: phenylalanine ammonia-lyase

PCR: Polymerase Chain Reaction

PEG: polyethylene glycol

PLD: phospholipase D

PtdOH: phosphatidic acid

P5CS: pyrroline-5-carboxylate synthetase

PLA: PHOSPHOLIPASE A1

PRO: Proline

ROS: reactive oxygen species

RWC: Relative water content

SA: salicylic acid

SDS: sodium dodecyl sulfate

SP1: stable protein 1

SOD: superoxide dismutase

SSPs: seed storage proteins

TBA: thiobarbituric acid

TDC: tryptophan decarboxylase

TFs: transcription factors

TM: turgid mass

TPS: Trehalose-6-phosphate synthase

TPP: Trehalose-6-phosphate phosphatase

TIP: tonoplast intrinsic protein

Zusammenfassung

Pflanzen haben die Fähigkeit, durch abiotischen und biotischen Stress verursachte Schäden zu lindern. Phytohormone spielen eine sehr wichtige Rolle bei der Anpassung an diese Arten von Stress. Um die Rolle von Jasmonat bei der Anpassung an osmotischen Stress zu erforschen, wurde eine Jasmonsäure (JA) - Biosynthesemutante (*cpm2*) von Reis, die in der Funktion der ALLENOXID CYCLASE (AOC) beeinträchtigt ist, und der entsprechende Wildtyp (WT) auf ihre Reaktionen nach Behandlung mit Polyethylenglycol (PEG) 6000 untersucht. Der WT zeigte Toleranz gegenüber osmotischem Stress, der einen vorübergehenden Anstieg von JA und JA-Isoleucin (JA-Ile) in Sprossen induzierte, der einem Anstieg der Abscisinsäure (ABA) voraus ging. In den Wurzeln war das Muster des hormonellen Anstiegs ähnlich, aber die Antwort schien schneller zu sein. In *cpm2*-Pflanzen wurden jedoch extrem niedrige Konzentrationen von 12-Oxophytodiensäure (OPDA), JA und JA-Ile nachgewiesen, unabhängig davon, ob sie mit PEG 6000 behandelt wurden oder nicht. In *cpm2*-Sprossen stieg ABA weniger an als bei WT, wenn sie osmotischem Stress ausgesetzt waren, während ABA-Level in Wurzeln etwas höher waren. Dies legt nahe, dass Jasmonate an der Regulation der ABA-Biosynthese als Antwort auf osmotischen Stress in Blättern beteiligt sind.

Bei osmotischem Stress könnte das erste hormonelle Signal eine Zunahme von Jasmonaten sein, und die Akkumulation von JA im frühen Stadium von osmotischem Stress könnte zu einem späteren Anstieg der ABA führen, da WT-Sprosse mehr ABA akkumulieren wenn sie osmotischem Stress ausgesetzt sind. In Reis scheinen jedoch beide hormonellen Wege mehr oder weniger unabhängig durch osmotischen Stress induziert zu werden, da Jasmonate früher als ABA und ABA sogar in Abwesenheit von JA induziert wurde.

Wenn Pflanzen osmotischem Stress ausgesetzt sind, ist die Funktion von JA in Sprossen und Wurzeln nicht genau gleich. Kurzfristig war der Anstieg der JAs in den

Wurzeln vorübergehend, während die JAs in den Sprossen im Laufe der Zeit akkumuliert wurden. Daher werden JAs hauptsächlich während einer ersten Reaktion auf diese Art von Stress gebildet. JAs werden genutzt, um molekulare Signale zu induzieren, die zur Anregung des Abwehrsystems in Wurzeln führen. Im Gegensatz dazu helfen JAs im Spross wahrscheinlich, dass sich die Pflanze durch bestimmte morphologische und physiologische Veränderungen an die raue Umwelt anpassen kann, um Wasser effizienter zu nutzen, da JAs ständig zunehmen, was mit dem Phänotyp der größeren Toleranz gegenüber osmotischem Stress korreliert. Auf längere Sicht kann JA zu einer Zunahme von Kronenwurzeln in Reaktion auf osmotischen Stress in Reis führen, was dazu beitragen kann, dass die Pflanzen mehr Wasser aus der Umgebung aufnehmen können und resistenter gegen osmotischen Stress werden.

Schließlich stellen wir auch verschiedene mögliche Mechanismen vor, um zu erklären, wie JA die osmotische Stressabwehr in Reis induziert. JA ist teilweise an der Regulation der ABA-Biosynthese in Reaktion auf osmotischen Stress in Blättern beteiligt. JA selbst und die Anreicherung von ABA induzierten das Schließen der Stomata, was in der Folge zu einer geringen Transpiration und Zurückhaltung von Wasser in den Blättern führen und das Ziel erreichen kann, sich an osmotischen Stress anzupassen. Zweitens hängt die ROS-Akkumulation mit dem endogenen JA zusammen, insbesondere kann der Anstieg von JA zu einer Abnahme des ROS-Gehalts unter osmotischen Stressbedingungen führen. Es ist zwar nicht klar, ob die Abnahme von ROS durch die blockierte Synthese verursacht wird, die aus JA resultiert, oder der Mechanismus der Eliminierung von ROS in Gegenwart von JA verstärkt ist. Wir denken, dass die Hemmung von ROS in WT die Pflanzen vor weiteren oxidativen Schäden bewahrt, da der Gehalt an Superoxid in *cpm2* sehr hoch und aller Wahrscheinlichkeit nach schädlich für Reis ist. Drittens korrelierte JA mit der Regulierung der Ressourcenverteilung in Pflanzen – insbesondere mit der Regulation einiger Aminosäuren und Sekundärmetaboliten, welche die Pflanzenresistenz gegenüber osmotischem Stress erhöhen könnten. Diese Funktion von JA kann jedoch das übermäßige Wachstum (wie *cpm2*) von Reis hemmen und

auch zum Schutz vor anderen Arten von biotischem und abiotischem Stress beitragen, da die meisten Aminosäuren und sekundären Metabolite eher der Verteidigung als dem Wachstum dienen. Letztendlich kann JA Pflanzen helfen, sich an den langfristigen osmotischen Stress anzupassen, indem sie die morphologischen Eigenschaften von Pflanzen, insbesondere die Konstruktion des Wurzelsystems, anpasst. Die Funktion von JA beim Schutz vor osmotischem Stress ist jedoch altersabhängig. Da die Verteilung von Ressourcen in jedem Stadium des Pflanzenwachstums unterschiedlich sein kann, kann auch die Anpassungsfunktion von JA an osmotischen Stress im Laufe der Zeit unterschiedlich sein.

Abstract

Plants have the ability to alleviate deleterious effects caused by abiotic and biotic stress. Phytohormones play a critical role in the adaptation to these stresses. To study the role of jasmonate in the adaptation to osmotic stress, a jasmonic acid (JA) biosynthesis mutant (*cpm2*) of rice, which is impaired in the function of ALLENE OXIDE CYCLASE (AOC) and its wild type (WT), was employed to investigate its responses after polyethylene glycol (PEG) 6000 treatment. WT showed tolerance to osmotic stress, which induced a transient increase of JA and JA-isoleucine (JA-Ile) prior to an increase in abscisic acid (ABA) in shoots. In roots, the pattern of hormonal increase was similar, but the response appeared to be faster. In *cpm2* plants, however, extremely low levels of 12-oxophytodienoic acid (OPDA), JA, and JA-Ile were detected, irrespective of whether or not they were treated with PEG 6000. In *cpm2* shoots, ABA increased less than in WT when exposed to osmotic stress, while ABA levels were slightly higher in roots. This suggests that jasmonates participate in the regulation of ABA biosynthesis in response to osmotic stress in leaves.

In addition, when exposed to osmotic stress, the first hormonal signal to osmotic stress might be an increase in jasmonates, and the accumulation of JAs in the early stage of osmotic stress might mediate a subsequent increase of ABA, as WT shoot accumulated more ABA when exposed to osmotic stress. However, in rice, both hormonal pathways seem to be induced by osmotic stress more or less independently, as jasmonates were induced earlier than ABA, and ABA was induced even in the absence of JA.

When plants are subjected to osmotic stress, the function of JA in shoots and roots is not identical. In the short-term, JAs' increase in roots was transient, whereas JAs accumulated in shoots over time. Thus, JAs are mainly used during a first reaction to this type of stress, JAs are utilized in order to induce molecular signals to arouse the defence system in root. In contrast, in the shoot, JAs probably assist the plant to adapt

to harsh environments through certain morphological and physiological changes towards adaption and more efficient water usage, as JAs constantly increase, which correlates with the phenotype of larger tolerance to osmotic stress. Over a relatively long time, JA may be related to crown roots increasing in response to osmotic stress in rice, which may help the plants absorb more water from the surroundings and obtain more resistance to osmotic stress.

Finally, we also propose several possible mechanisms to explain how JA induces osmotic stress defence in rice. JAs partially participate in the regulation of ABA biosynthesis in response to osmotic stress in leaves. JA itself and the accumulation of ABA induced stomatal closure, which may subsequently result in low transpiration and reservation of water in leaves, and accomplish the goal of getting used to osmotic stress. Secondly, ROS accumulation is related to endogenous JA, specifically, the increase of JA may lead to a decrease in ROS content under the condition of osmotic stress. It is not clear, however, whether the decrease of ROS is caused by the blocked synthesis which results from JA or that the mechanism of elimination of ROS is enhanced in the presence of JA. We think that the inhibition of ROS in WT prevents plants from further oxidative damage, as the content of superoxide in *cpm2* is very high and, in all probability, is harmful to rice. Thirdly, JA is correlated with the regulation of resource allocation in plants. Specifically, JA is associated with the regulation of some amino acids and secondary metabolites, which might increase plant resistance to osmotic stress. However, this function of JA may inhibit the excessive growth (just like *cpm2*) of rice and may also contribute to defense against other kinds of biotic and abiotic stress, as most amino acids and secondary metabolites are used for defense rather than growth. Finally, JA may help plants to adapt to long-term osmotic stress by adjusting the morphological features of plants, especially the construction of root systems. However, the function of JA in the defense of osmotic stress is age-dependent. Since the allocation of resources may be different at each stage of plant growth, the adjustment function of JA to osmotic stress may also differ over time.

1. Introduction

1.1 Plant stress

Plants are widely distributed in the natural world, and the environments in which they grow are always complex. Even in the same area, environmental conditions vary greatly over a year or one day, which makes it difficult for plants to survive. However, as plants do not have legs and cannot escape from harmful environments, they must withstand and adapt to these conditions (Nick 2013). When the extent of environmental variation is too serious and exceeds the range that a plant requires for its normal growth and development, it will become an adverse environmental factor, which is termed *stress* (Dobson and Smith, 2000). Environmental stress initiates a series of physiological metabolic reactions in plants, and is mainly manifested as an impediment of metabolism and growth (Kranner et al., 2010). In severe cases, it even leads to death of the entire plant. There are many types of stress, including drought, cold, high temperature, waterlogging, salinity, pests, and environmental pollution (Cheeseman, 2007). Among them, drought, salinity, and extreme temperatures are the most common abiotic stresses, and the adverse effects on plants are also most prominent (Mittler, 2006). These abiotic and biotic stresses may dehydrate cells, destroy the membrane system, increase cell membrane permeability, and disorder various enzyme activities and metabolic activities on the membrane (Fujita et al., 2006). They may also reduce the photosynthetic rate and affect the respiratory rate. Therefore, in order for plants to complete their life history, it is necessary to overcome these unfavorable conditions and adapt to the environment in which they live (Yang et al., 2009). Since stress factors can affect many stages of plant growth and development and several stress factors sometimes affect plants at the same time, the processes of tolerance and defense of plants to stress are complex (Mittler, 2002). Currently, with extant changes in global climate, plants' survivability has been put to severe tests, and their adaptation mechanisms to the adverse survival environment, as well as anti-reverse mechanisms, have gradually attracted great attention from researchers in the bioscience field (Herrero and Zamora, 2014).

1.2 Plants responses to Abiotic stresses

In order to adapt to the environment and have a higher probability of survival, during the long-term process of evolution, plants have also gradually formed a variety of defense mechanisms to resist the restraints of adverse factors on their growth and development. These mechanisms are broadly existent at the plants' cellular levels, molecular levels, as well as the entire organism's level (Harris, 1975). Concerning physiological and biochemical aspects: the cell filtration rate is reduced with stomatal closure, so as to maintain tissue tension and water contents (Yu et al., 2006); adapt to stress through changes in leaf morphology and structures, as well as changes in the root-shoot ratio and root density; and resist stress injuries through alterations of the expression patterns of the adversity stress-related genes and regulations of the relevant metabolic pathways (Kasuga et al., 1999; Wang et al., 2003; Yin et al., 2009), in order to resist the environment stress. On the cellular level, when plants encounter adversity stress, they trigger the relevant response reactions, causing increases in the contents of such substances as sugars, organic acids, soluble proteins, proline, and ABA (Xiantao et al., 1998). They can also generate new homodimerization or alter the ingredients of the membrane lipid to resist and avoid harms brought about by the adversity factors (Bohnert et al., 1995).

In addition to the above reactions, some molecules that are able to respond to the adversity stress signals may also appear in the plant cells in order to conduct regulations of the expressions of the stress-related gene (Liu et al., 2001). This then causes increases or declines of the expression volumes of the relevant proteins, and further regulates the physiological and biochemical process of the plant's resistance to adversity (Viña, 2002). From the perceptions of the stress signals, to the gradual transfers and transductions, and then to the conduction of responses, plants have evolved a series of anti-adversity mechanisms (Chen and Zhu, 2004; Kim, 2007).

1.3 Drought stress

Water is the paramount environmental factor that impacts plants' growth, and it is also one of the most important limiting factors on crop yields (Bradford and Hsiao, 1982). In our study, we focus on the role of jasmonic acid in response to water deficiency. Thus, in the following, we will mainly introduce two kinds of stress: drought and osmotic stress. Drought has become the most critical ecological challenge faced by our current world, and is also the crucial factor limiting the sustainable development of agriculture (Allen et al., 2010). The types of plant response to drought stresses mainly include the drought-avoidance type and the drought-tolerant type (Mart ńez-Ferri et al., 2000). The drought-avoidance type means that, when under a drought stress condition, plants can maintain a high-water potential by extending and expanding root systems, avoiding tissue injuries by increasing water absorption in the deeper soil layer, reducing evapotranspiration water consumption, and delaying tissue dehydration (Mart ńez-Ferri et al., 2000). For example, some desert woody plants have developed root systems, deep burials and big root-crown ratios, and thus can sufficiently absorb and utilize moisture in deep soil. Some plants even possess characteristics of small leaf cells, thinner cellular walls, mechanical tissues of the thick walls, denser leaf veins, transduction tissues, and a high degree of keratinizing of thicker waxy layers (Lee et al., 2007). The drought-tolerant type means that plants elevate their tolerance to droughts through a series of physiological reactions, the main models of which include: low osmotic potential prior to water stress, and the conducting of osmotic regulations and turgor pressure maintenance during stress periods (Gaxiola et al., 2001).

Beginning in the last century, people have noted the adverse influences of water deficiency on plants' photosynthesis. They also discovered that, during the process of drought stress, plants' physiological changes, especially photosynthesis, are one of the most representative indicators (Cornic, 2000; Flexas et al., 2004; Reddy et al., 2004; Chaves et al., 2009). Stomatal closure was thought to be the most important factor

limiting photosynthesis during drought stress, as it can decrease CO_2 deficiency (Cornic, 2000; García-Mata and Lamattina, 2001; Chaves et al., 2009). Under different water deficit conditions, there are two different types of limitations: stomatal limitation and non-stomatal limitation (Ni and Pallardy, 1992; Escalona et al., 2000). When exposed to a slight water deficit condition, photosynthesis decline is caused by stomatal closure; when the water deficit is more severe, photosynthesis declines could be attributed to other factors (e.g., non-stomatal limitation) (Escalona et al., 2000).

1.3.1 Responses of Morphology and Anatomical Features to Drought Stresses

Under water deficit conditions, plants mainly focus on retaining more water and enhancing water-use efficiency for adaptation (Loveys et al., 2004). During the process of leaf development, when suffering from water stress, the enlargement of cells is firstly inhibited, thus causing the leaf area to be smaller than that under normal water conditions (Marron et al., 2003), and transpiration and water dissipation are subsequently reduced (Miyashita et al., 2005). Therefore, the reduction of the leaf area is a type of plant strategy for adaptation to drought stress (Anyia and Herzog, 2004). Meanwhile, water stress elevates the rates of leaf senescence and death, thus reducing water dissipation (Munné-Bosch and Alegre, 2004). Leaf wilting and curling are also effective ways to reduce water dissipation (Wang et al., 2012). Turner (1986) studied the relationship between leaves' structures and water utility rates under drought conditions through the leaves' anatomical structures, and proved that the smaller the mesophyll cells under the unit leaf area, the higher the water-use efficiency. In addition, under drought stresses, the leaf epidermis will be thickened, which can elevate the plants' slight reflective power, weaken transpiration, elevate plants' drought resistance, and reduce mechanical damage during wilting (Schulze, 1986).

1.3.2 Responses of Root Morphology to Drought Stresses

The plants' root system is the main organ for direct absorption of water, and it plays an indispensable role in plants' drought tolerance (Passioura, 1981). Under a drought

condition, the changes of the roots should be beneficial to the absorption of as much moisture in the soil as possible (Chen et al., 2011). The plant root system adapts to the stress of drought via various means, such as extensions in horizontal or vertical directions, growth of root lengths and density, the development of fine roots with stronger absorption functions, and elevation of the root-shoot ratio (Pace et al., 1999). During the process of experiencing drought, the reductions of root water potential and turgor pressure will always result in shrinkage of cortical cells and increases of the root lengths and root-shoot ratios (Huang and Fry, 1998). When plants are under drought stress, adaptive changes will occur in the xylem, so as to elevate the moisture's flowability between the roots and the leaves (Alvarez et al., 2008).

1.3.3 Physiological and Biochemical Adaptations to Drought Stress in Plants

In order to cope with drought stress, plants will generate a series of physiological and biochemical changes, in order to make resistance reactions, including stomatal regulations, PH regulations, osmotic adjustments, and scavenging of reactive oxygen species (ROS) (Chaves and Oliveira, 2004). When plants encounter drought stress, they will instinctively adjust their metabolic activities, elevate the concentration of intracellular solute and reduce osmotic potential, thus decreasing water potential, maintaining the ability of continued water absorption from external soil with lower water potential and maintaining turgor pressure, thus enabling metabolic activities to be maintained at a relatively more normal state and level (Singh et al., 1972; Irigoyen et al., 1992; Reddy et al., 2004). The accumulation of intracellular solute will lead to the decline of osmotic potential (Sakthivelu et al., 2008). These solute substances are known as *osmotic adjustment substances* and can be approximately categorized into two major kinds: (1) organic substances, such as dissociation proline, sugar, protein, and endogene hormone (Mohammadkhani and Heidari, 2008; Sperdouli and Moustakas, 2012); and (2) inorganic ions, such as Na^+, K^+, etc. (Ghoulam et al., 2002; Patakas et al., 2002). By accumulating these small-molecule osmotic regulation substances, plants elevate osmotic pressure inside their bodies, so as to maintain intracellular turgor pressure and water potential, prevent dehydration, and maintain

cells' normal physiological functions (Hoekstra et al., 2001; Ramanjulu and Bartels, 2002). Drought stress interferes with the plants' antioxidant enzyme system, causing an increase of oxygen free radicals within the plants and the function of membrane lipid peroxidation, thus resulting in injuries of the membrane system (Cruz de Carvalho, 2008; Noctor et al., 2014). There exist some antioxidants within the plants, such as superoxide dismutase (SOD) and peroxidase (POD), and these antioxidants feature the function of eliminating superoxide radicals, thus reducing the harms of oxidants on the membrane system and protecting the plant bodies (Chaves et al., 2003). Meanwhile, in the case of drought stress, abscisic acid (ABA) content within the plants is increased (Zhang et al., 2006). ABA can obviously reduce leaves' moisture evaporation, decrease membrane permeability of the leaf cells, increase the soluble protein content of the leaf cells, induce the formation of the protective enzymes of the biomembrane system, reduce the peroxidation degree of the membrane lipid, protect the integrity of the membrane structure, increase the plants' antioxidant capacity under adversity stress, and further elevate the plants drought resistance (Boyer, 2009; Parent et al., 2009; Pinheiro and Chaves, 2010).

1.3.4 Genetic Regulations of Plant Adaptations under Drought Stress

Seki et al. (2002) studied the model plant *Arabidopsis thaliana* using microarrays, and the results indicated the following: when water stress occurs within the plant's cells, the expressions of many genes are activated or inhibited, and these genes may participate in at least four function groups: signal transduction, transcription regulation, cellular metabolism and transport, and the protection of the cell structures. Under drought stress, the analysis of the microarrays of the rice genome indicated that, among the 30 expressed sequence tags (ESTs) in rice, 30% of the genes were obviously up-regulated, while 18% were down-regulated (Galli et al., 1994).

According to the effects of products of stress-inducible genes, they can be divided into two kinds (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 2000). The coding products of the first kind of genes play a

direct role: 1) functional proteins preventing the cells from moving away from damages of water stress, such as LEA protein, osmotic protein, antifreeze protein, aquaporin, ion channel protein, chaperonin, mRNA binding protein, etc. (Ingram and Bartels, 1996; Ohno et al., 2003; Mazel et al., 2004); and 2) osmotic factors, such as proline, betaine, and synthesis of certain sugars (Quan et al., 2004). Under stress, aquaporin, sugar transporters, and proline transporter proteins transform moisture, sugar, and proline by plasma membrane and vacuole, in order to adjust osmotic pressure inside and outside of the cell (Sakamoto and Murata, 1998; Abebe et al., 2003; Baud et al., 2004). Moreover, toxic degradation enzyme, such as glutathione S transferase, soluble epoxide hydrolase, superoxide dismutase, catalase, and ascorbate peroxidase, can prevent cells from reactive oxygen damage (Kovtun et al., 2000). So far, the specific functions of this kind of gene products have received wide attention and been studied thoroughly, including using LEA protein gene (Ohno et al., 2003), proline synthase gene (Zhu et al., 1998), betaine synthetase gene (Sakamoto and Murata, 1998), etc., which obtained some transgenic plants with drought and salt resistance.

The coding products of the second kind of genes participate in controlling expressions of genes downstream and signal transduction, including: 1) protein kinase inducing and transforming stress signals, such as mitogen-activated protein kinase (MAPK) that activates mitogen, and calcium-dependent protein kinase (CDPK), which depends on calcium, receptor protein kinases, ribosomal-protein kinase, transcription-regulation protein kinase, etc. (Torii, 2000; Becraft, 2002; Di évart and Clark, 2003; Haffani et al., 2004; Yoshida and Parniske, 2005); 2) transcription factors that transduct signals and control gene expressions, such as bZIP transcription factors (transcription factor family with leucine zipper), MYC transcription factors (helical transcription factor family with basic helical strings), MYB transcription factors (transcription factor family with tryptophan strings), AP2/ERF transcription factors, etc. (Chen et al., 2002; Tang et al., 2005); and 3) proteases that play an important role in signal transcription, such as acid phosphatase and phosphatase C (Luan, 2002;

Mayrose et al., 2004).

Under water deficiency stress, two major regulation routes exist: the ABA-independent pathway and the ABA-dependent pathway (Yang et al., 2011). The ABA-independent pathway is mainly regulated by the transcription factor family containing APETALA1 DNA structure domains and dehydration response element binding (DREB) (Barrero et al., 2006). In contrast, NAC, AREB/ABF, and MYB transcription factors mainly participate in the ABA-dependent signal transduction pathway (Fujita et al., 2004).

(1) ABA-dependent Pathway

ABA-responsive gene expression is regulated by transcription factors (TFs) which bind to cis-elements in the promoter regions upstream of their target genes (Shinozaki et al., 2003). Most ABA-regulated genes contain ABA-responsive elements (ABREs; PyACGTGG/TC) as the determinant cis-elements in their promoters (Busk and Pagès, 1998; Hattori et al., 2002; Zhang et al., 2005; Gómez-Porras et al., 2007). In the ABA-dependent pathway, bZIP-type ABFs/AREBs (ABRE-binding or responsive factors) (Choi et al., 2000; Yoshida et al., 2010; Xu et al., 2013), AP2/ERF (Mizoi et al., 2012), MYB, MYC, NAC, and bHLH were identified as positive regulators (Abe et al., 2003; Ding et al., 2014; Nakashima et al., 2007). As an ABA-independent regulator, DREB2 protein, a member of the AP2/ERF family (Sakuma et al., 2006; Yoshida et al., 2014), is able to activate the expression of target genes encoding proteins involved in osmoprotection and metabolism (Abe et al., 2003; Ding et al., 2014; Nakashima et al., 2007). In addition, SNAC1-regulated protein phosphatase gene OsPP18 also mediates osmotic and oxidative stress response through the ABA-independent pathway (You et al., 2014; You et al., 2012).

Through regulating the activity of the protein kinases bZIP (basic region-leucine zipper) TFs, and target gene promoters with contents of ABRE cis-acting elements,

regulation of expressions of the ABA-dependent downstream genes is achieved (Choi et al., 2000; Seki et al., 2007). For instance, drought can induce expressions of transcription factors, such as bZIP, MYB, and MYV, augmenting the combination with downstream recognition sites, and induce expressions of target genes (Wada and Shinozaki, 1997; Abe et al., 2003). In the ABA's endogenous deficient mutant *los5*, the expressions of drought response genes, such as P5CS, COR15A, RD29A, and RD22, are inhibited greatly (Xiong et al., 2001). Extant literature also reported that activation of ABA-dependent signals necessitates ABFs/AREBs transcription factors, and such two cis-acting elements of DRE and ABRF-like are critical during the regulation process of the induction and expression of the RD29A gene (Narusaka et al., 2003). In rice, the ABI5-like1 (ABL1) protein is a bZIP family transcription factor, and expression of ABI1 can be induced by abiotic adversity conditions, including ABA, drought, and high salinity. Moreover, ABI1 can combine directly with ABA response elements G-box and ABREs, and mutant *abi1* exhibits a phenotype that is insensitive to ABA (Fig. 1.1) (Yang et al., 2011).

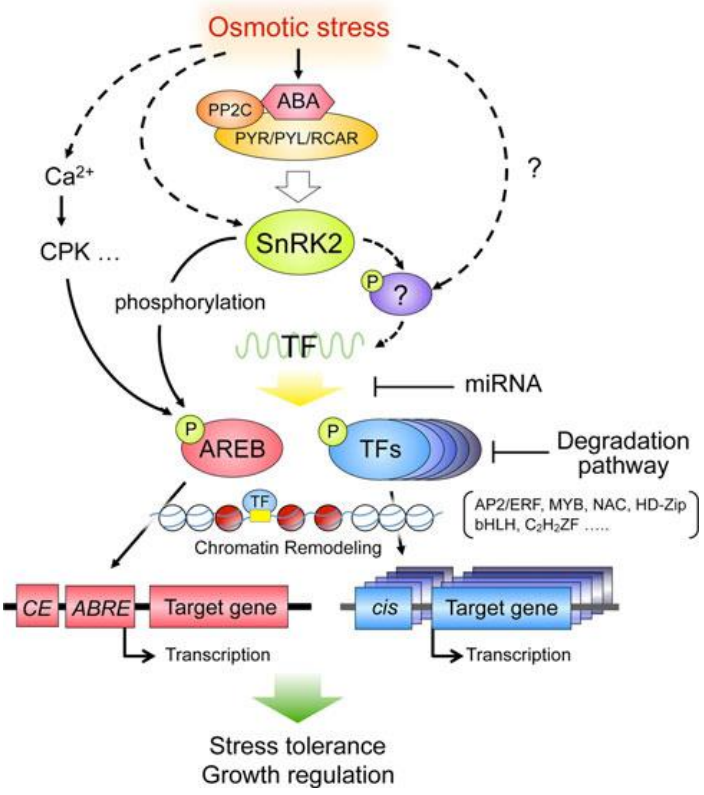

Figure 1.1. Model of ABA-mediated transcriptional regulation in response to osmotic stress. PYR/PYL/RCAR ABA receptor-PP2C complexes regulate SnRK2-AREB/ABF pathways to control major ABA-mediated ABRE-dependent gene expression. TFs control ABA-mediated gene expression through interacting with cis-elements in promoter regions upstream of the target genes as a reaction to water stress during vegetative growth in *Arabidopsis*. Broken lines indicate conceivable, but not definitive, routes. TF, transcription factor; PP2C, protein phosphatase 2C (Fujita et al. 2011).

(2) ABA-independent Pathway

The expression of ABA-independent pathway induction genes during drought stress is mainly regulated through the cis-acting elements of drought response element (DRE); under a drought condition, AP2-like transcription factors DREB2B and DREB2A regulate drought response genes, which contain DRE cis-acting elements (Sakuma et al., 200). DREB1/CBF4 can be induced by drought and ABA. The excessive expression of *CBF4* gene in *Arabidopsis thaliana* can induce rapid up-regulation expressions of the stress response genes with contents of CRT/DRE elements, thus augmenting the resistance of transgenic *Arabidopsis thaliana* to drought and low temperature (Haake et al., 2002). Current research indicates that there are actually no strict boundaries in the ABA-dependent and ABA-independent pathways, and these two pathways have numerous crossings in the processes of signal transduction (Wathugala et al., 2011). Previous study has revealed that the domains of these gene protein promoters mostly contain DRE cis-acting elements, and induce the expressions of DREB1/CBF under low temperatures (Narusaka et al., 2003). In the endogenous ABA-deficient mutant, DREB1/CBF is still subject to the strong induction of low temperature stress (Narusaka et al., 2003). Under the inductions of temperatures and drought, DREB2A exhibits an ABA-independent expression model (Fig. 1.2). The excessive expressions of the DREB1 and DREB2 genes in *Arabidopsis thaliana*, and rice enable the plants to exhibit resistance to low temperatures and drought (Dubouzet et al., 2003; Wang et al., 2008).

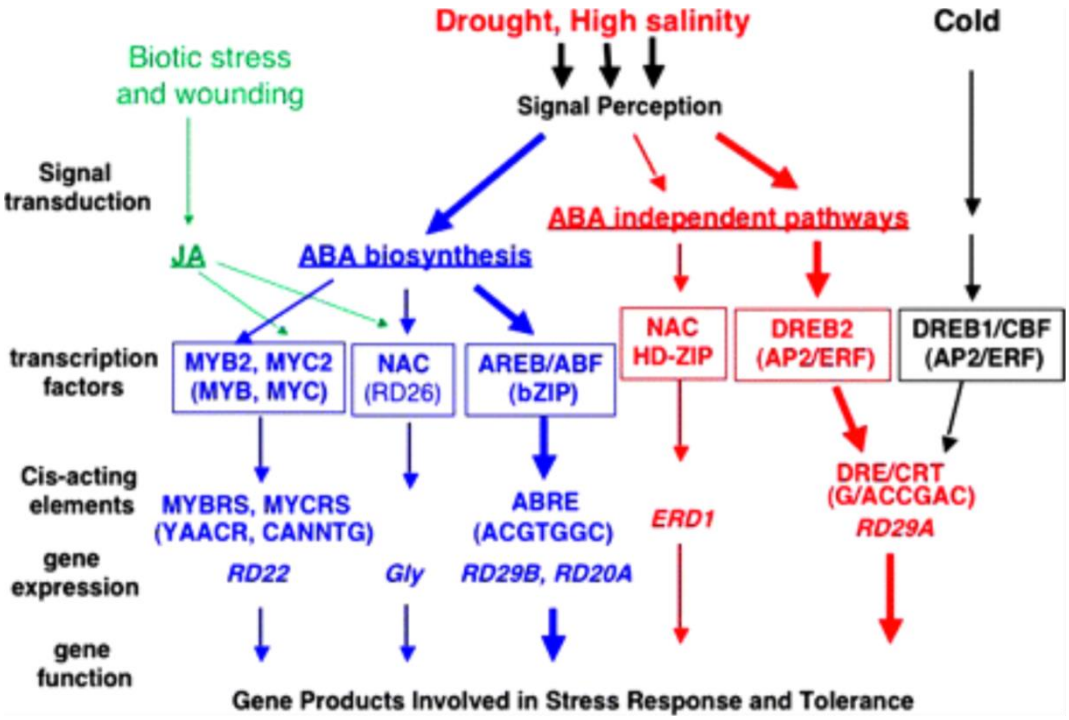

Figure 1.2. Transcriptional regulatory networks of abiotic stress signals and gene expression. A minimum of six signal transduction pathways exist in high salinity, drought, and cold-stress responses. Three are ABA-independent, and three are ABA-dependent. In the ABA-dependent pathway, ABRE serves as an ABA-responsive element. AREB/ABFs are AP2 transcription factors that participate in this process. MYC2 and MYB2 function in ABA-inducible gene expression of the RD22 gene. MYC2 also participates in JA-inducible gene expression. The RD26 NAC transcription factor is involved in JA- and ABA-responsive gene expression in stress response. These NAC and MYC2 transcription factors may function in cross-talk during abiotic-stress and wound-stress responses. In one of the ABA-independent pathways, DRE is mainly involved in the regulation of genes, not only by salt and drought, but also through cold stress. DREB1/CBFs participate in cold-responsive gene expression. DREB2s are essential transcription factors in dehydration and high salinity stress-responsive gene expression. Another ABA-independent pathway is controlled by drought and salt, but not by cold. The HD-ZIP and NAC transcription factors participate in ERD1 gene expression (Shinozaki et al 2007).

1.4 Osmatic Stress

Osmotic stress has been conceived as mere water scarcity; in fact, however, it

represents a complex syndrome comprising at least three components that can occur individually or in different combinations: ionic stress (salinity), water scarcity stress (drought), and nutrient-depletion stress (alkalinity) (Riemann et al. 2015). In nature, osmotic stress always appears independently, and the closest stress is drought stress. However, drought stress is more than osmotic stress. For example, when the soil lacks water, it will become harder and the soil temperature will be higher. Therefore, although osmotic stress is similar to drought stress, osmotic stress should not be equated with drought stress.

1.4.1 How Do Plants Respond to Osmotic Stress

Plants have evolved a complete sensory perceptual system to cope with osmotic stress, and reduce the harms of osmotic stress to the lowest level by different measures (Verma et al., 2016). In early osmotic stress, it mainly comprises activation of the Ca^{2+} signal channel and the production of secondary signal molecules (Denis and Cyert, 2002; Lee et al., 2004). These secondary signal molecules include inositol phosphate, reactive oxygen species (ROS), activation of kinase cascades, etc. (Verma et al., 2016). Osmotic stress can limit the growing rate of plants, which constitutes the main factor leading to yield reduction for crops (Rangel et al., 2008; Rahnama et al., 2010).

When plants suffer from osmotic stress, the main way of losing moisture is through gas exchanges by stomas on the blade surface (Sarwat and Tuteja, 2017). The opening and closing of stomas are controlled by outside environmental factors, such as CO_2 level, illumination, and biotic and abiotic stresses (Araújo et al., 2011). In fact, the opening and closing of stomas are mainly regulated by changes of guard cells' turgor pressure (Luan, 2002). Moreover, the opening and closing of stomas influence plant photosynthesis and transpiration, thus adjusting moisture in plants (Hetherington and Woodward, 2003). While stomata adjustment is one of the mechanisms of plant defense against osmotic stress (Rahnama et al., 2010), under osmotic stress, most

stomas on the lower epidermis of plant leaves deform and sink under nearby epidermal cells (Skirycz et al., 2011). CO_2 and H_2O to be stuck in leaves may provide some benefits. When the outside environment changes, CO_2 that was stuck in folds could make the concentration of CO_2 around stomas increase and provide sufficient resources for photosynthesis, and could also prevent moisture inside of cells from dissipating (Lawson et al., 2003).

1.4.2 Adjustments of Osmotic Balance by Soluble Substances

When subject to osmotic stress threat (caused by drought, salinity, etc.), plants maintain osmotic pressure by the synthesis of a variety of osmotic adjustment substances (Burg et al., 1996). These substances are mainly some non-toxic molecules, such as amino acids, glycine betaine, soluble sugars and alcohols, and some poly compounds (Bartels and Sunkar, 2005). When plants are subject to drought (Choudhary et al., 2005) and/or high salinity (Yoshiba et al., 1995), the contents of proline will be obviously accumulated. The proline in plants is mainly obtained by glutamate from the three-step reaction from glutamate-semialdehyde (GSA), pyrroline-5-carboxylate synthetase (P5CS), and P5C reductase (P5CR) (Szabados and Savoure, 2010). The metabolic enzyme of proline includes proline dehydrogenase/ early response to dehydration (PDH1/ERD5), PDH2, P5C dehydrogenase (P5CDH), and ornithine-delta-aminotransferase (OAT) (Szabados and Savoure, 2010). In most plants, P5SC has two genes, while P5CR only has one gene (Verbruggen et al., 1993; Strizhov et al., 1997). In *Arabidopsis thaliana*, the expression modes of the two P5CS genes differ. P5CS2 is a constitutive expression (Verbruggen et al., 1993; Strizhov et al., 1997); whereas, the expression of P5CS1 is induced by osmotic stress, and it is induced by oxidative stress mediated by ABA-insensitive 1 (ABI1) (Yoshiba et al., 1995; Strizhov et al., 1997). Proline can not only serve as a kind of raw material for protein synthesis, but it can also regulate expressions of genes related to proline metabolism (Satoh et al., 200). In addition, it can eliminate single oxygen and maintain ROS-eliminating enzymes, such as peroxide enzyme and

glutathion-S-transferase (Székely et al., 2008; Islam et al., 2009). In addition, it can also maintain the electronic balance of electronic transmission chains (Chaves et al., 2009). Proline can elevate the plants tolerance to osmotic stress, such as some halophilic plants, including *Thellungiella halophila* and *Lepidium crassifolium* (Murakeözy et al., 2003; Taji et al., 2004). However, some plants, such as *Camphorosma annua* and *Limonium spp*, resist osmotic stress not by relying on proline, but on sugar-like osmotic substances (Taji et al., 2004; Gagneul et al., 2007).

When different plants are under stress condition (including, but not limited to, osmotic stress), many non-constitutive carbohydrates, such as glucose, sucrose, trehalose, and some sugar alcohols will accumulate, and these sugars provide the plant cells with 50% of the osmotic pressure (Ashraf and Harris, 2004). These sugars may, as osmotic adjustment substances, protect the activity of some macromolecules and the structures of the cell membranes (Mansour, 2000; Ashraf and Harris, 2004; Manchanda and Garg 2008). Trehalos can be synthesized in insects and many lower plants; however, in higher plants, besides some resurrection plants, the content of trehalose is very low (Yang et al., 2010). The trehalose-6-phosphate synthase (TPS) and trehalose-6- phosphate phosphatase (TPP), which represent the trehalose synthetic genes in rice, can augment trehalose content and elevate tolerance to osmotic stress (Jang et al., 2003).

Formed in photosynthesis, mannitol is a substance which has the features of the functions of osmotic adjustments (Loescher et al., 1992). Some research has indicated that mannitol can protect Thioredoxin, Ferredoxin, and Glutathione (Mikołajczyk et al., 2000). Mannitol-1-phosphate dehydrogenase (M1PDH) genes, which are expressed in tobacco and rice, enhance tolerance to osmotic stress in plants (Tarczynski et al., 1993; Abebe et al., 2003). In *Coffea Arabica*, there are three key enzymes: Mannose-6-phosphate reductase (Ca M6PR), Phosphomannose-isomerase enzyme (Ca PMI), and Mannitoldehydrogenase (Ca MTD), which control the synthesis of mannitol (Carvalho et al., 2014). These three genes can respond to

drought, high salinity, and high temperatures (Carvalho et al., 2014).

1.5 Jasmonate Signaling System in Plant Defense System

Plant hormones are active substances that are generated by plant cells after they receive inductions of the specific environmental signals, and can regulate the plants' physiological responses at low concentrations (Bari and Jones, 2009). Plant hormones participate in the regulation of various aspects and stages of plant growth, including the determination of the stem cell niche during the process of embryo development, organ development and growth during the process of postembryonic development, seedling germination, and fruit ripening (Wolters and Jürgens, 2009; Santner et al., 2009). Until now, 10 kinds of hormones or analogues have been reported, which are, respectively: auxin, gibberellin (GA), cytokinin, brassinolide (BR), abscisic acid (ABA), ethylene, jasmonic acid (JA), salicylic acid (SA), nitric oxide (NO), and strigolactone (Santner and Estelle, 2009). In recent years, an in-depth understanding has been obtained of the processes of synthesis, decomposition, transportation, and signal transductions of most plant hormones (Leveau and Lindow, 2005). In the following, we will briefly introduce jasmonic acid, which is one of the most critical defense hormones.

1.5.1 History of Jasmonate

As a kind of new plant endogenous hormone, jasmonic acid (JA) broadly exists in organisms (Rao et al., 2000). JA, along with a series of compounds with similar biological activities as the volatile methyjasmonic acid (MeJA) and amino acid derivatives, are known as jasmonates (JAs). Free JA was first separated from the culture liquid of *Lasiodiplodia thebromae* in 1971 as a kind of plant growth inhibitor (Aldridge et al., 1971). MeJA was first refined from the essential oil of *Jasminum grandiflorum* L. (Demole et al., 1962), and it is the main aromatic substance of flowers. Therefore, as an essential composition of perfumes, it is used in quantity in the production of perfumes (Pichersky and Gershenzon, 2002). Usually, there are high

contents of JA in the top of stems, leaves, immature fruit, and root tips (Hause et al., 2002). In addition, its contents in reproductive organs, especially fruits, are higher than those in such nutritional organs, such as leaves, stems, and buds (Creelman and Mullet, 1995).

1.5.2 JA Biosynthesis

Oxylipins constitute a large family of oxygenated fatty acids. Biotic and abiotic stresses activate lipases that release unsaturated fatty acids, inducing oxylipins biosynthesis. One of the most commonly known oxylipins is jasmonic acid. A reaction catalyzed by lipoxygenase (LOX) enzymes participates in the formation of fatty acid hydroperoxides, which is a critical step in oxylipin biosynthesis. In *Arabidopsis*, six LOX genes have been identified, four of which encode 13-LOX enzymes. 13-LOX enzymes catalyze the initial step in the biosynthesis of jasmonic acid (Bertoni 2012). Linolenic acid is mostly present in the form of esterified glycerolipid, and not in the form of free fatty acid. Jasmonic acid is biosynthesized from α-linolenic acid (α-LeA/18:3) in the octadecanoid pathway (Wasternack, 2007; Wasternack and Hause, 2013; Huang et al., 2017). α-LeA is synthesized from dienoic fatty acid by the coordinated actions of FATTY ACID DESATURASE (FAD) and PHOSPHOLIPASE A1 (PLA) in plastids. Then, α-LeA is oxygenated by a 13- lipoxygenase (13-LOX) to a 13-hydroperoxyoctadecatrienoic acid (13-HPOT). 13-HPOT is sequentially converted to 12,13(S)-epoxyoctadecatrienoic acid (12,13-EOT), and the naturally occurring enantiomer (9S,13S)-12-oxo-phytodienoic acid (OPDA) by the catalysis of ALLENE OXIDE SYNTHASE (AOS) (Park et al., 2002; von Malek et al., 2002) and ALLENE OXIDE CYCLASE (AOC) (Ziegler et al., 2000), respectively. OPDA is further transported to peroxisomes via transporter COMATOSE (CTS1) (Theodoulou et al., 2005; Sharma and Laxmi, 2016), and subsequently reduced to 3-oxo-2-(cis-2′-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0) in a reaction catalyzed by OPDA REDUCTASE (OPR). OPC-8 is then activated to OPC-8 CoA by ACYL-COENZYME A SYNTHETASES (Huang et al., 2017). The carboxylic acid side chains of CoA esters are then shortened to jasmonoyl-CoA by three rounds of

β-oxidation catalyzed by three enzymes: ACYL-CoA OXIDASE (ACX), multifunctional protein (MFP), and L-3-KETOACYL-CoA THIOLASE (KAT) (Schneider et al., 2005). Jasmonoyl-CoA is subsequently cleaved by THIOESTERASE (TE) to form cis-7-iso-jasmonicacid [(+)-7-iso-JA] (Sharma and Laxmi, 2016) (Fig. 1.3).

1.5.3 JA Metabolism

(1) JAR1

JA can conjugate to amino acids catalyzed by JASMONATE RESISTANT1 (JAR1), and this reaction may be critical in the metabolism of JA. JAR1 is a member of the GRETCHEN HAGEN3 (GH3) gene family, and this gene family is involved in auxin conjugation (Staswick and Tiryaki 2004). The role of JAR1 was determined to be significant upon identification of (+)- 7-iso -JA-Ile, as JAR1 is the most bioactive compound among more than 40 JA compounds (Fonseca et al. 2009). JAR1 is a jasmonoyl amino acid conjugate synthase, which creates an acyl-adenylate/thioester intermediate through the use of (+)-7-iso -JA as a substrate. Most structure-activity relationships, which have been studied for numbers of JA-dependent responses over the previous three decades (for review cf. Wasternack 2007), can be now be elucidated. JA-Ile and JA accumulate in a nearly 10:1 ratio in numerous high plants. (+)-*7-iso*-JA, which is the initial product of JA biosynthesis, has long been thought to epimerize to the more stable (-)-JA. Moreover, (-)-JA was considered to be the indicator of endogenous increase of JAs resulting from environmental stimuli. Levels of JA-Ile and JA, however, are normally recorded without individual enantiomorph detection.

(2) Methyl Jasmonate

JA can be metabolized to many derivatives, several of which are involved in the defense-signaling system in plants. Among them, methyl jasmonate (MeJA) plays a critical role in signaling systems (Wasternack 2007). MeJA functions as a volatile in the air in interplant communication, and constitutes a strong signal (Farmer and Ryan

1990). Jasmonic acid carboxyl methyltransferase (JMT) participates in the conversion from JA to MeJA (Seo et al. 2001). Enhanced pathogen resistance has been reported in *Arabidopsis*. Augmented stress resistance has also been found when overexpressing JMT, suggesting that MeJA plays a defense-signaling role (Seo et al. 2001). However, it has been shown that the biological activity of MeJA was apparent only when MeJA was converted to JA followed by its conjugation to JA-Ile (Stitz et al. 2011).

(3) Jasmonoyl-Isoleucine

The JA amino acid conjugate (+)-7-iso -JA-Ile (jasmonoyl-isoleucine, JA-Ile) is involved in defense signaling (Staswick et al. 1998; Staswick and Tiryaki 2004; Kang et al. 2006; Katsir et al. 2008; Gfeller et al. 2010 ; Kombrink 2012 ; Wasternack and Hause 2013). In the above, we introduced JAR1, and this enzyme might be involved in JA-Ile biosynthesis (Hause et al. 2000; Méndez-Bravo et al. 2011; Westfall et al. 2012). JA-Ile can be hydrolyzed to release JA and Ile (Kazan and Manners 2008). The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) conjugated to JA gas also been reported in plant tissue. An unidentified conjugating enzyme is suggested to participate in this process (Staswick and Tiryaki 2004). Synthesis of JA-ACC constitutes a regulatory mechanism for the availability of ACC and JA for conversion to active ethylene and JA-Ile, respectively (Staswick and Tiryaki 2004), indicating interconvertibility of the different forms of JA.

1.5.4 Jasmonate Receptor Complex in JA Signal Perception

The identification of COI1 (*CORONATINE INSENSITVE1*) was found in coronatine-insensitive mutants in *Arabidopsis*, and it is a key factor in jasmonate perception and signal transduction pathway. The *Arabidopsis* invalid mutant for COI1 (*coronatine insensitive 1*) is very insensitive to jasmonates (Feys et al. 1994; Xie et al. 1998). COI1 has been considered to play an essential role in JA signal recognition (Yan et al. 2009). Moreover, it was demonstrated to be a receptor of jasmonates (Gfeller et al. 2010). The gene COI1 is requisite for almost all JA-mediated responses

(Devoto et al. 2005; Balbi and Devoto 2008). In addition, the *Arabidopsis* coi1 mutant exhibited defects in nearly all JA-dependent functions (Kazan and Manners 2008).

The COI1 encodes an F-box protein which is part of an E3 ubiquitin ligase complex, which is involved in ubiquitin-mediated protein degradation (Balbi and Devoto 2008; Wager and Browse 2012). COI1 protein associates with RBX1 (*RING-BOX* PROTEIN1), CUL1 (*CUL*LIN1), and Skp1-like proteins ASK1 (ARABIDOPSIS S-PHASE KINASE-ASSOCIATED PROTEIN 1) and ASK2 to form SCF^{COI1} (SKP1, F-box COI1, CDC53p/CUL1) ubiquitin ligase complex in *A. thaliana* (Devoto et al. 2002; Xu et al. 2002; Pauwels and Goossens 2011; Zhang et al. 2012c).

1.5.5 JA Signaling Pathway

JA receptor JAZ proteins have been determined as an inhibitory factor of jasmonate signaling (Chini et al. 2007; Thines et al. 2007). JAZ proteins inhibit JA-responsive gene expression (Chini et al. 2007; Howe 2010; Wasternack and Hause 2013). Moreover, the transcriptional repressor, JAZ, has been reported as an essential component in the receptor complex participating in JA signal (Sheard et al. 2010). Twelve (12) JAZ proteins have been found in *Arabidopsis thaliana* (Chung and Howe 2009; Pauwels and Goossens 2011; Wager and Browse 2012).

The *COI1* gene is required for JA-mediated defense response against pathogens (Xie et al. 1998). SCF^{COI1} is the key regulator in the JA signaling pathway for ubiquitination, and subsequently it will be degraded by 26S proteasome (Kawamura et al. 2009). The ubiquitin-proteasome-mediated proteolysis is involved in the jasmonate signaling system (Xu et al. 2002; Feng et al. 2003) (Fig. 1.3).

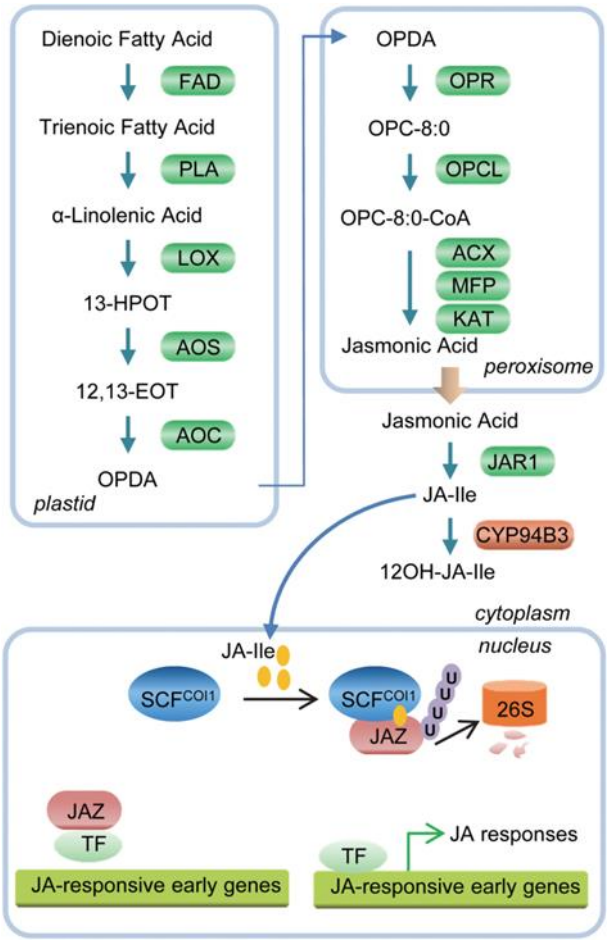

Figure 1.3. An illustration of jasmonate (JA) signaling and biosynthesis. JA-Ile, which is biosynthesized by JA biosynthetic enzymes in plastids, the cytoplasm, and peroxisomes, can be inactivated by CYP94B3. JA-Ile causes the interaction of CORONATINE INSENSITIVE1 (COI1) with JA ZIM-domain (JAZ) family proteins, leading to the degradation and ubiquitination of JAZ proteins through the 26S proteasome. Consequently, downstream transcription factors (TFs) are de-repressed, enabling their activation of JA-responsive early genes and JA responses. ACX, acyl-CoA oxidase; AOC, allene oxide cyclase; AOS, allene oxide synthase; 12,13-EOT, 12,13(S)-epoxyoctadecatrienoic acid; FAD, fatty acid desaturase; 13-HPOT, 13-hydroperoxyoctadecatrienoic acid; JA-Ile, jasmonoyl-L-isoleucine; 12OH-JA-Ile, 12-hydroxy-JA-Ile; JAR1, JASMONATERESISTANT 1/jasmonate-amido synthetase; KAT, 3-ketoacyl-CoA thiolase; LOX, 13-lipoxygenase; MFP, multifunctional protein; PLA, phospholipaseA1; OPC-8:0, 3-oxo-2(cis-2′-pentenyl)-cyclopentane-1-octanoic acid; OPCL, OPC-8:0 CoA ligase; OPDA, (9S,13S)-12-oxo-phytodienoic acid; OPR, OPDA reductase (Huang et al., 2017).

1.5.6 Function of Jasmonate

JA can help plants' defense against such biological stresses as pests (Chehab et al., 2012), fungi (Lee et al., 2001) and plant viruses (Shang et al., 2011), and abiotic stresses, including high temperature (Lin-Wang et al., 2011), low temperature (Kondo et al., 2005), high salinity (Yoon et al., 2009), and high illumination (Wierstra and Kloppstech, 2000). In addition, JA also plays an important role in the process of plants' normal growth and development, such as a series of physiological activities, including embryo differentiation (Ruduś et al., 2006), stomatal movements (Suhita et al., 2004), pigment synthesis (Rudell and Mattheis, 2008), seed germination (Linkies and Leubner-Metzger, 2012), growth of shoots and roots (Kang et al., 2005), seedling growth (Heijari et al., 2005), pollen development (Wilson and Zhang, 2009), fruit maturation (Kondo et al., 2000), development of trichome (Traw and Bergelson, 2003), vine curing (Larronde et al., 2003), and senescence process (Tamogami and Kodama, 2000) (Fig. 1.5).

Among such functions of jasmonic acid, the most noticeable are its capabilities in the defense against diseases and pests (Robert-Seilaniantz et al., 2011). Under the adversity of pests, plants activate expressions of defense genes inside of the bodies through JA signals and induce the plant to generate various kinds defense chemicals, such as alkaloids, polyphenol oxidase (ppo) and protease inhibitor, in order to elevate the plant's defense abilities to damage caused by pests (Turner et al., 2002). Therefore, jasmonic acid is also considered to be a defense hormone that has resistance to pests and diseases (Farmer and Ryan, 1992; Kramell et al., 1995). JA can not only regulate bacterial infections of plants resulting in the expressions of many adversity genes (Zhao et al., 2003), but it can also engage in crosstalk with other plant hormone signals, for instance, jasmonic acid signal pathways in the processes of plant adversity response and development interact with ethylene signal pathways (Lorenzo et al., 2003; Charlton et al., 2005). As indicated by microarray analysis, the 41 JA response genes also participate in the signal pathways of ethylene, auxin and salicylic acid,

which suggests that the JA signal pathways and other hormone signal pathways have some crosstalk (Robert-Seilaniantz et al., 2011).

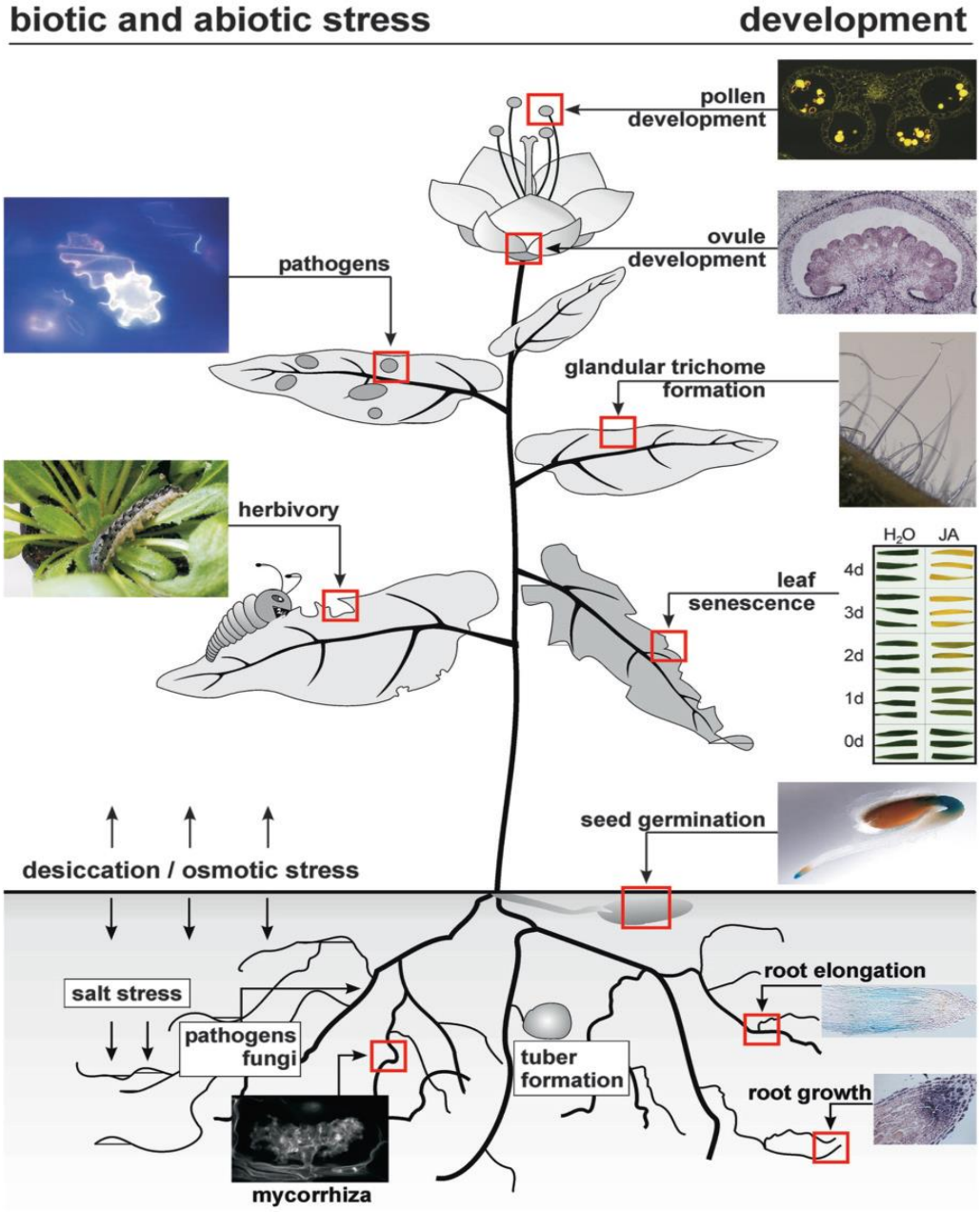

Figure 1.5. Illustration of jasmonates in plant development (*right*) and plant responses to biotic and abiotic stress (*left*). Stress responses are determined by a hypersensitive response upon pathogen attack, by arbuscular mycorrhiza, and by herbivory on *Arabidopsis*. The function of jasmonates in development is indicated by a cross-section of anthers of *Arabidopsis* revealing the release of pollen, through immunocytochemical detection of allene oxide cyclase in a cross-section of tomato ovules, by seedling growth and root elongation of a tomato seedling showing allene oxide cyclase promoter activity via GUS staining, by senescing barley leaf

segments upon treatment with jasmonate, through root growth revealing immunocytochemical detection of the allene oxide cyclase protein in the root tip, and by trichomes. In addition, jasmonates are also involved in adventitious root formation, lateral root formation, attack by nematodes, light signaling, freezing tolerance, and growth inhibition (Wasternack 2014).

1.6 Rice

Rice (*Oryza sativa* L.) is one of the major staple food crops globally. It plays a vital role in economics and food security, and provides food for more than half of the world's population, especially in Asia (Dorosh, 2001). The latest research has indicated that the world's population will continue to grow. By 2050, it is predicted to reach 9 billion (Godfray et al., 2010) and, at that time, our world will need to have a 70%-100% increase in grain to meet the demands of this population increase (Baulcombe et al., 2009). Over the past half a century, the grain output has more than doubled, with the ratio of the global starving population substantially decreasing (Shomura et al., 2008). Nevertheless, since the world population has nearly tripled, in 2009, the world's undernourished population surpassed 1 billion for the first time (Godfray et al., 2010).

As the world's main grain crop, rice, accounts for 1/3 of the farming areas of grain crops (Fageria et al., 2010), contributing 40% of overall calories for human beings (Cheng et al., 2007). Indeed, approximately 50% of the population relies on rice as its staple food (Meharg et al., 2009). From 1960 to 1989, the world's total rice output has been increasing at an annual rate of 3.3%; after 2000, it has been increasing at an annual rate of 0.8% (Peng et al., 2000). The annual growth rate of total rice yield has obviously declined, failing to meet the demands of population growth (Kushibuchi, 1997; Normile, 2008). Many scholars, based on the demands of the world's population for grain, estimated that only when the annual growth rate of global rice total yield is at 1.2% (during 2001-2030) will the constantly increasing demands of the population be satisfied (Yuan, 2004; Van Nguyen and Ferrero, 2006).

1.7 Scope of this study

In rice, jasmonates can mediate many developmental processes and stress responses (Syatyna and Riemann, 2012; Liu et al., 2015; Dhakarey et al., 2016). Our previous investigation showed that in OPDA- and JA-deficient rice *allene oxide cyclase (aoc)* mutants, salt stress tolerance was increased (Hazman et al., 2015). Kurotani et al. (2015) also demonstrated a similar phenotype in plants overexpressing a jasmonate-inactivating enzyme. Similarly, the AOC mutant *coleoptile photomorphogenesis 2 (cpm2)* exhibited more tolerance for drought stress (Dhakarey et al., 2017). It has been shown that lack of jasmonate leads to differential accumulation of reactive oxygen species (ROS; Hazman et al., 2015; Dhakarey et al., 2017) and altered cell wall architecture (Dhakarey et al., 2017). Does this mean that jasmonate deficiency causes higher abiotic stress tolerance?

As salt stress is composed of multiple factors (e.g., ionic, osmotic stress), and drought is often accompanied by other stresses (e.g., hard soil, heat), it is worth determining how rice plants would respond to a single stress factor, such as osmotic stress. We therefore employed the AOC mutant *cpm2* (Riemann et al., 2013) to study polyethylene glycol (PEG) 6000 induced osmotic stress in rice. These insights can assist us to know more about the function of JA in defense against osmotic stress. It may also help us to improve drought tolerance of rice cultivars which are otherwise osmotic-sensitive in humid climates.

Central questions are:

1. What are the functions of JAs in the response to osmotic stress?

In our laboratory, *Oryza sativa* L. ssp. japonica cv. Nihonmasari (WT) and jasmonate-deficient genotype *cpm2* were employed. Different concentrations of PEG 6000 were applied to induce osmotic stress on these two types of rice. It was found that normal rice showed more tolerance to osmotic stress. When exposed to strong osmotic stress (25% PEG), WT retains more water in rice leaves and exhibits a

relative healthy condition compared to *cpm2*. Under a low osmotic stress condition (5%-20% PEG), *cpm2* showed more mass and length loss compared with rice under a normal condition. This means that, when treated with PEG, the growth rate in *cpm2* decreased more compared with WT. All of the experiments showed that osmotic stress has more of a negative impact on rice with low endogenous jasmonic acid. The result indicated that, as a stress defense hormone, JA contributes to defense in biotic and abiotic stress in most cases, and thus JA generally functions as a positive regulator of stress tolerance.

2. As a signal, how does JAs contribute to defense against osmotic stress? What are JAs' different roles in rice shoots and roots?

In many studies, JA was regarded as a stress defense hormone, which is a signal that can induce further physiological response to abiotic and biotic stress. In our present work, we artificially induced osmotic stress on WT and *cpm2*, and then measured the content of several jasmonic acid related hormones, as well as the expression of some JA-dependent genes in different time points and in rice shoots and roots. When treated with 25% PEG, jasmonates were strongly and rapidly accumulated in rice shoots and roots. However, jasmonate accumulation in roots preceded that in shoots. Moreover, this increase in roots was transient, while jasmonates accumulated in shoots over time. The result of gene expression in rice roots was also slightly earlier than in shoots. In both rice shoots and roots, both hormone and gene expression appeared several hours after PEG treatment, which indicated that jasmonates constituted a short-time signal. We propose that, in roots, jasmonates are utilized in order to induce certain morphological and molecular changes towards adaption and more efficient water usage. On the other hand, in the shoots, jasmonates probably help the plant to adapt to harsh environments, as they are constantly increasing, which correlates with the phenotype of larger tolerance to osmotic stress.

Taken together, regulation of hormonal content and expression of related genes correlated well. It is found that roots probably use jasmonate signals for immediate adaption, while shoots utilize hormone for sustained signalling during continuous

stress, culminating in a hyper-accumulation as an indication of damage under prolonged severe stress conditions.

3. How does JAs contribute to defense against osmotic stress through morphological and physiological changes? What is the possible mechanism for JA to contribute to defense against osmotic stress?

As previously discussed, JAs generally function as a positive regulator of osmotic stress tolerance. It is also known that jasmonates probably assist the plant to adapt to harsh environments as a short-time signal. However, how does this signal work in subsequent steps and what is the potential mechanism of the stress defense? To answer this, we measured some physiological indicators after PEG treatment in our later experiment. In the result, we found that *cpm2* accumulated less ABA in the shoot and, as a consequence, the decrease of stomatal conductance in *cpm2* was less than in WT. So, one of the functions of JA in defense against osmotic stress is to regulate stomatal conductance. The stomatal conductance might be directly regulated by JA itself and indirectly regulated by JA through accumulation of ABA. In addition, most measured amino acids accumulated less in *cpm2* compared with WT, and several amino acids related to secondary metabolite synthesis. Thus, JA may correlate with the biosynthesis of secondary metabolites and help plants to adjust to osmotic pressure and better adapt to osmotic stress. Furthermore, JA promotes the growth of crown root, which further results in the increase of the surface area of roots, and could also be one mechanism for why JA augmented resistance to osmotic stress in rice, as the increased crown root is very likely to assist plants to absorb more water.

2. Materials and Methods

2.1 Plant materials, growth, and stress conditions

In this study, *Oryza sativa* L. *ssp. japonica* cv. Nihonmasari was used as a WT, and the AOC mutant *cpm2* (Riemann et al., 2013) was employed as a jasmonate-deficient genotype. The rice seeds were dehusked and surface sterilized by incubation in 70% ethanol for 1 min followed by brief washes with double-distilled water. Ethanol incubation and washing were repeated three times. Subsequently, the seeds were incubated in a sodium hypochlorite solution containing ~5% of active chlorine for 20 min followed by three washing steps in sterilized double-distilled water. The seeds were sown on 0.4% phytoagar medium (Duchefa, The Netherlands), which contained 0.344 g/L murashige and skoog basal salt mixture (MS). The seeds were germinated in a culture room (at 25 °C, continuous light of 120 μmol $m^{-2}s^{-1}$) for 6-7 d, and then transferred to custom-made sterilized floating racks and moved to a glass container containing double-distilled water and 0.344 g/L MS for 1-2 d adaptation. Subsequently, half of the seedlings was transferred to a 25% solution of PEG 6000 to induce osmotic stress, and the other half was transferred to water as a control. Shoots and roots were harvested separately, frozen in liquid nitrogen, and stored at -80 ℃ until use for phytohormone or gene expression analyses.

2.2 Analysis of morphology parameters

Samples used for morphology parameters were cultured in phytoagar medium for 7 d, then transferred to double-distilled water, and cultured for an additional day. Seedlings were treated with different concentrations of PEG 6000 (0, 5%, 10%, 15%, 20%) for 3 d. Both shoot and root length were measured using a scale with precision of 1 mm. A balance with the precision of 0.0001 g was used for the measurement of shoot and root biomass.

2.3 Measurement of stomatal conductance (Gs)

Plants were cultured in 0.4% phytoagar medium which contains 0.344 g/L MS for 7 d, and subsequently cultured in 0.344 g/L MS solution for 21 d. Leaves of rice samples were used for the measurement of stomatal conductance using a leaf porometer (SC-1, Decagon Devices, Pullman, Washington, U.S.A.).

2.4 Measurement of relative water content (RWC) of shoots

Shoots of plants of all treatments were used for the determination of RWC. First, fresh mass (FM) was determined. The leaves were then placed in distilled water inside of a closed 50 ml Cellstar® polypropylene tube. After 12 h, samples were gently wiped with tissue paper to remove water from the leaf surface, and turgid mass (TM) was measured. In the last step, samples were placed in a drying oven at 70 ℃ for 72 h followed by determination of dry mass (DM). RWC was calculated according to the equation: (RWC,%)=[(FM - DM)/(TM - DM)] * 100. Five biological replicates were used for each treatment.

2.5 Endogenous levels of ABA, OPDA, JA, and JA-Ile

OPDA, JA, JA-Ile, and ABA were quantified simultaneously from approximately 50 mg of tissue using a standardized ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)-based method according to Balcke et al. (2012). [$^2H_5$]OPDA, [$^2H_6$]JA, [$^2H_2$]JA-Ile, and [$_2H_6$] ABA (50 ng each) were added as internal standards.

2.6 RNA extraction and quantitative real-time PCR

We used the InnuPrep plant RNA kit (Analytika Jena RNA kit) to isolate total RNA from the shoots of control and osmotic stressed plants (25% PEG 6000, 1 h and 3 h) according to the manufacturer's instructions. The Spectrum Plant Total RNA kit (Sigma-Aldrich RNA kit) was used to isolate total RNA from the roots of control and osmotic stressed plants (25% PEG 6000, 1 h and 3 h) according to the manufacturer's instructions. The mRNA was transcribed into cDNA using the M-MuLV cDNA

Synthesis Kit (New England Biolabs; Frankfurt am Main, Germany) according to the instructions of the manufacturer. The RNase inhibitor (New England Biolabs; Frankfurt am Main, Germany) was used to protect the RNA from degradation. The amount of RNA template was 1 µg. cDNA synthesis was performed with a M-MuLV cDNA Synthesis Kit (New England Biolabs; Frankfurt am Main, Germany) according to the instructions of the manufacturer.

Real-time PCR analysis was carried out in 20 µL reaction tubes containing a final concentration of 200 nm of each primer, 1X GoTaq colourless buffer, 200 nm of each dNTP, 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 1:10 cDNA dilution according to Svyatyna et al. (2014). The primer sequences for the genes of interest are listed in Table 1.

Table 1. Sequences of forward and reverse primers for the genes of interest and the housekeeping gene used for normalization.

Gene name	Accession no.	Forward (5'-3' prime)	Reverse (5'-3' prime)
OsUBQ5	AK061988	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT
OsJAZ1	Os04g0653000	CAGCAGGTTGGTGAGCAAAG	TCCATCCCTGATGCTTCCAT
OsJAZ4	Os09g0401300	GAGTGCCAATGACAACAAGTCATC	TGATTCGTCGCGGTTGCT
OsJAZ11	Os03g0180900	CAGCCTTGCCTACCAGACATG	GACGATCCTGTTCTTCCTCTTCTC
OsAOS1	OS03G0766900	GCCCGGTCATCTTATTTTCC	TGCAACTCCGTATCCGTACA
OsAOS2	OS03G0225900	GGAGGAAGCTGCTGCAATAC	GGAGGTTGAAGCTTTGGTGA

OsAOC	OS03G0438100	TGCCTCAACAACTTCACCAACTA	CACATGCCGCAATTAACACTAAA
OsOPR7	Os08g0459600	CTCAACCACCGGTTTCCTCA	TCCATGCATCAGTCTGCTCT
OsJAR1	Os05g0586200	AGGAGGCATCAAAGTTCCTGG	CTCAGCTCCCAGAAGATCACG
OsLEA3	Os05g46480	TCACTTCAAATTCGGTGCAA	CACACCCGTCAGAAATCCTC
OsP5CS	Os05g0455500	GAAGTGGTAATGGTCTTCTC	AGCAAATCTGCGATCTCATC
OsPAL4	Os02g41680	CTT CAC AAC AGC TAA TCG AG	CGC ACT CCA TTT CAG TAC CA
OsTDC1	Os08g04540	GCG AGG GTG AAA CCT TCC A	GCG AGC CGG TGG AGT CC
OsTDC2	Os07g25590	GTG CTG CCT TTA ACA TTG G	CAT GTC ATT GGA CTT TGC TAT CTG T

2.7 Measurement of malondialdehyde (MDA)

Levels of malone dialdehyde (MDA) in shoots and roots were determined using the thiobarbituric acid (TBA) method as described by Hodgson and Raison (1991) with minor modifications. Briefly, shoots and roots were collected in 2 ml reaction tubes, their frews masses determined, and then shock-frozen in liquid nitrogen. After addition of a sterilized steel bead (5 mm) to the tube, the frozen tissue was ground twice by a Tissuelyser (Qiagen, Hilden, Germany) at 23 Hz for 45 s. 1 ml 0.1 M phosphate buffer (pH 7.4) was then added to the tube, followed by centrifugation at 8000 × g for 10 min. 200 µl of the supernatant was then transferred to a mixture containing 750 µl of 20% (v/v) acetic acid, 750 µl of 0.8% (w/v) TBA, 200 µl of Milli-Q water, and 100 µl of 8.1% (w/v) sodium dodecyl sulfate (SDS). As a blank, 200 µl of supernatant was replaced by 200 µl volume of 0.1 M phosphate buffer.

Subsequently, reaction mixtures were incubated at 98 ℃ for 1 h. After cooling down to room temperature, the absorbance at 535 nm was measured in a UV-Vis spectrophotometer (Uvikon XS, Goebel Instrumentelle Analytik GmbH, Germany) and corrected against the absorbance at 600 nm monitoring an unspecific background. Lipid peroxidation could then be calculated as μM·g FW^{-1} MDA from A535 to A600 using an extinction coefficient of 155 mM^{-1} cm^{-1}. Data represent mean values and standard errors from four independent experimental series.

2.8 Measurement of superoxide

Rice shoots and roots used for superoxide measurements were 10-d old and treated with 25% PEG 6000 for 24 h. $O_2^{\cdot-}$ in rice shoots and roots was estimated using the nitroblue tetrazolium (NBT) method as described by Vacca et al. (2004) with minor modification. Samples were collected in 2 ml reaction tubes, frozen in liquid nitrogen, and homogenized as described above. After adding 100 mM sodium-phosphate buffer (pH 7.2) containing 1 mM diethyl dithiocarbamate to the reaction tubes for inhibiting SOD activity, samples were centrifuged at 12,000× g for 20 min, and amounts of superoxide anion ($O_2^{\cdot-}$) were determined in the supernatant. Change of A530 in the supernatant was measured over the 1^{st} min in the spectrophotometer. Calculation of specific activity was calculated by the formula $C=A_{(change)}/12.8$, assuming an absorption coefficient of 12.8 mM^{-1} cm^{-1} (Murphy et al., 1998). C is the concentration of solution, $A_{(change)}$ is the change of A530, and 12.8 mM^{-1} is the coefficient.

2.9 Gas chromatography-mass spectrometry (GC-MS) analysis

Samples used for gas chromatography-mass spectrometry (GC-MS) parameters were cultured in phytoagar medium for 7 d, then transferred to double-distilled water, and cultured for an additional 3 d. Young plants were treated with 25% of 6 h and 12 h. Samples were analyzed according to the method used to create FiehnLib (Kind et al. 2009).

2.10 Data analysis

The significance of the difference of most data between the control and PEG 6000 treatment was analyzed by a student's t-test using PASW Statistics 18.0 software (Macintosh, SPSS Inc., Chicago, IL, U.S.A.). Some of the data (ABA, OsLEA3, Gs) had to be analyzed regarding the difference between control and treatment, and WT and *cpm2* were analyzed by one-way analysis of variance (ANOVA). Once a significant difference was detected, *post-hoc* LSD multiple range tests at $p < 0.05$ were used to identify statistically significant differences. The results shown in the graphs are presented as the mean value ± standard error.

3. Results

3.1 Compared with *cpm2*, WT adapted to osmotic stress of different concentrations better. JA plays a positive role in defending against osmotic stress.

To investigate the effect of JA in rice osmotic stress, we used *Oryza sativa* L. *ssp*. *japonica* cv. Nihonmasari (WT) and jasmonate-deficient genotype mutant *cpm2* in our experiments. Through treating both genotypes of rice with polyethylene glycol 6000 (for convenience, all polyethylene glycol 6000 will hereafter be abbreviated as PEG), we observed the reactions of WT and *cpm2* towards osmotic stress, respectively. To determine whether JA plays a positive or negative role in osmotic stress, the optimal method is to observe the degree of water loss of rice after PEG treatment. The most intuitive way is to determine the degree of wilting and curling of rice leaves. Therefore, we firstly performed PEG pretreatment to WT and *cpm2*, and the concentration of PEG was divided into six gradients from 5% to 30% (5%, 10%, 15%, 20%, 25%, 30%). In the initial experiment, we found that when the concentration of PEG was less than 20% (including 20%), we could hardly observe the phenomenon of water loss of the leaves whether on WT or on *cpm2*. Even if we extended the treatment to 3 d, it was still difficult to discern the obvious change of moisture of the leaves of the plant. When treated with 25% PEG, however, both genotypes of rice showed the phenotype that they could not adjust to high-intensity osmotic stress in a short period of time. In 24 or 18 h after treatment, we found that both genotypes of rice (WT and *cpm2*) leaves lost a substantial amount of water. Even 6 h after treatment, a certain degree of curling of leaves was observed. When the PEG concentration reached 30%, the effects on both rice conditions were similar to that on 25%. Considering that 30% PEG is hard to dissolve completely, we used 25% PEG in most of the following experiments.

3.1.1 *cpm2* is more sensitive to high-intensity (25% PEG) osmotic

stress

After culturing WT and *cpm2* in phytoagar medium for 7 d, we cultured them in water for 3 d. We then treated the 10-d rice with 25% PEG. After 18-h treatment, WT was almost still in a fairly healthy condition, except that there was a slight curling on the top of the third leaf (Fig. 3.1A). In contrast, we could see that for *cpm2*, either the second or the third left showed severe water loss (Fig. 3.1A). When we extended the treatment to 24 h, WT also exhibited a certain degree of water loss, but the wilting was obviously more severe on *cpm2* (Fig. 3.1B). Then, we observed the changes of the second and the third leaves, respectively; the third leaf of *cpm2* was totally wilted, while WT wilted a half degree. The second left of *cpm2* also wilted to a great degree, while the second of WT only had a certain degree of wilt on the tip. From this, we could find that, compared with *cpm2*, WT obviously had a strong ability to defend against osmotic stress, especially high-intensity osmotic stress. Therefore, it can be concluded that JA plays a positive role in rice defense against osmotic stress. Meanwhile, in the same plant, it was more difficult for younger leaf tissue to defend against osmotic stress than riper and older ones in most cases. This phenomenon exists in both WT and *cpm2*, and may have to do with the distribution of secondary metabolites of plants in different plant tissue. This issue will be discussed in detail in the following.

Apparent images can only provide a qualitative judgment. To determine the reactions of two kinds of rice after being treated with osmotic stress quantitatively, we measured changes in water content of rice leaves. Concerning the 10-d plant of rice, after treating it with 25% PEG for 24 h, we measured changes in relative water content (RWC) of the biggest leaf and the third leaf. It was found that, under normal circumstances, the water content of rice leaf is approximately 95%. When being treated with osmotic stress for 24 h, the relative water content of WT leaves hardly decreased, while it reduced to nearly 30% in *cpm2*, and the water loss rate reached more than 60%. This is close to the circumstance of 18 h in Fig. 3.1A. By measuring

the water content of leaves, WT is further demonstrated to have greater resistance to osmotic stress.

From the qualitative result of images and the quantitative result of relative water content, it is obvious that under a strong osmotic stress condition (25% PEG), WT showed more tolerance to stress.

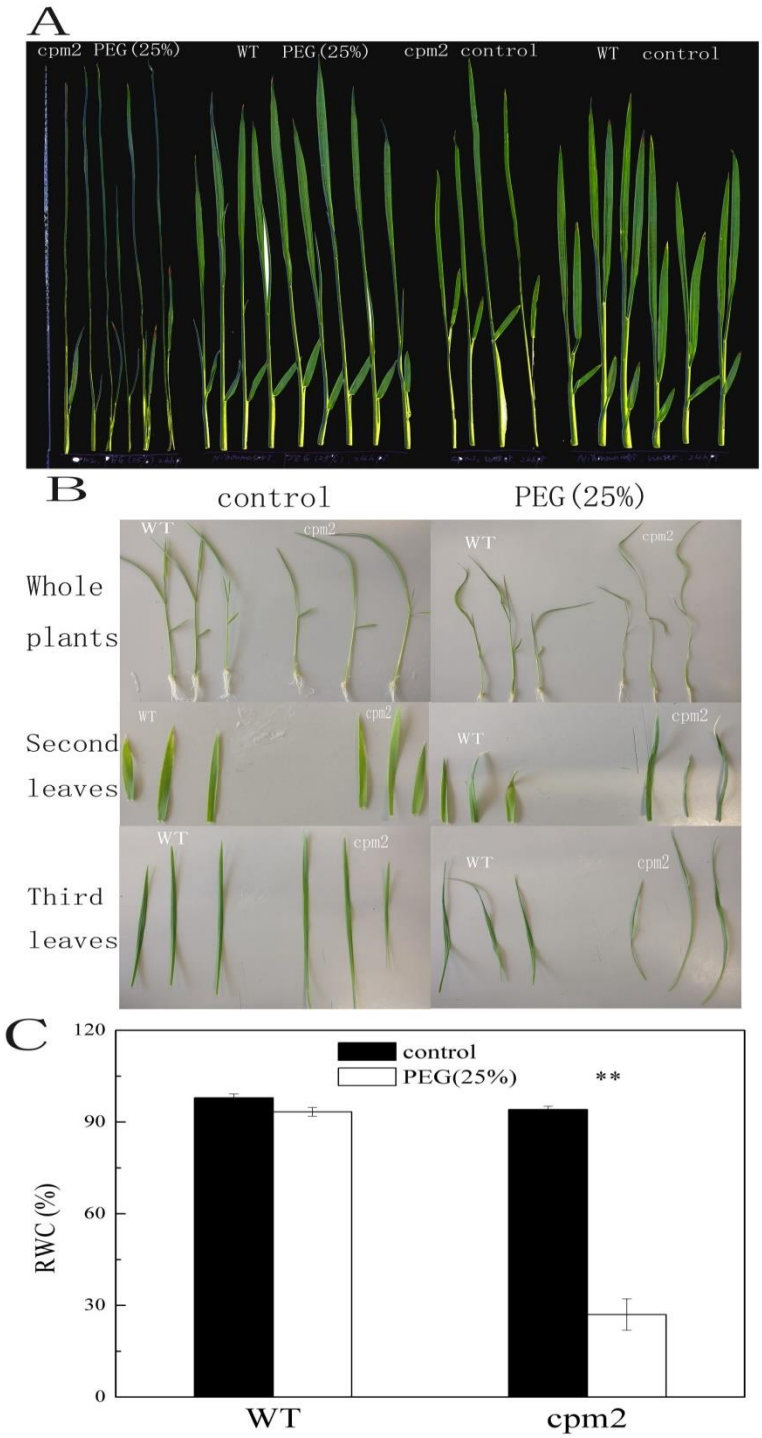

Figure 3.1. Effects of osmotic stress on morphology, RWC, and stomatal conductance of wild

type (WT) and JA biosynthesis mutants (*cpm2*). (A) Ten-day-old rice was subjected to osmotic stress at a high concentration (25% PEG) for 18 h. (B) Ten-day-old rice (including whole plant, second leaf, and third leaf) was subjected to a 25% PEG solution for 24 h. (C) Relative water content (RWC) of 10-day-old rice that was subjected to a 25% PEG solution for 24 h. Values are means ± SE of eight plants. Asterisks indicate statistically significant differences between the control and 25% PEG treatment in two genotypes (WT and *cpm2*) (student's t test; *P < 0.05 and **P < 0.01).

3.1.2 *cpm2* is more sensitive to low-intensity (≤20% PEG) osmotic stress in morphology

Through the experiments above, it was proven that WT can show much greater resistance than *cpm2* when suffering from strong (25%) osmotic stress. As stated previously, it is difficult to judge resistance in two genotypes through the degree of water loss, when WT and *cpm2* are suffering from low-concentrations of osmotic stress (PEG≤20%). However, in order to compare the different reactions of WT and *cpm2* under a low degree of osmotic stress, we performed another experiment. We divided 10-d-old rice (WT and *cpm2*) into five groups. Each group was treated with 0% (only H_2O), 5%, 10%, 15%, 20% of PEG, respectively. After 3-d treatment, some main morphological indexes of rice were measured, including root and shoot length, root and shoot mass, and the quantity of crown roots. As shown in Fig. 3.2A, the sizes of the two genotypes of rice (WT and *cpm2*) reduced with the increase of PEG concentration. Shoot length and root length of WT also decreased basically with the increase of PEG concentration. Moreover, shoot and root mass increased slightly under 5% PEG, and then reduced greatly with the increase of PEG concentration (Fig. 3.2B-E). Shoot length and root length of *cpm2* increased slightly under 5% PEG condition, and then reduced with the increase of PEG concentration. Shoot mass had few changes before PEG concentration reached 10%, and it started to reduce obviously from 15% (PEG). Root mass also showed few changes before 5% PEG, and started to reduce obviously from 10% to 20% (PEG concentration) (Fig. 3.2B-E).

Overall, in both WT and *cpm2*, the length and mass of rice root and shoot all exhibited a downward trend with PEG concentration increasing. This kind of trend became apparent as the PEG concentration reached 10%. In addition, we could also find that, in the case of normal growth (0% PEG), these morphological indexes of *cpm2* were always more than those of WT. In other words, in the case of normal growth, *cpm2* always grew more rapidly than WT. This may have to do with hormone balance in plants, which will be discussed in the following. This phenomenon could also be seen visually in our images (Fig. 3.2A). Although all of the morphological indexes (shoot and root length, shoot and root mass) in *cpm2* were always higher than in WT in the normal condition (0% PEG), the gap became closer (smaller) with the increase of PEG concentration. Until it reached 20%, the gap of these indexes between WT and *cpm2* was no longer significant ($p>0.05$). This indicated that, even when the osmotic stress was not that strong, it still had a greater influence on *cpm2* than on WT. Overall, it was found that WT could better adapt to low-concentration osmotic stress (PEG≤20%).

Besides the length and mass of roots and shoots, the quantity of crown roots after being treated by different concentrations of PEG (0-20%) was measured. It was found that the quantity of crown roots was not very sensitive to PEG in *cpm2*, and it increased slowly with the increase of PEG concentration. In contrast, the quantity of crown roots of WT changed greatly. Under the 10% PEG condition, the quantity of crown roots in WT was more than in *cpm2* ($p<0.05$), and in other concentrations no major differences were observed ($p>0.05$) (Fig. 3.2F). The changes of crown roots in different PEG concentrations indicated that JA might help plants better adapt to osmotic stress through an increase of crown roots. This is because, normally, the increase in the quantity of roots always coincides with the extension of roots' surface area and the increase of water absorption ability. In addition, WT is indeed more resistant than *cpm2* under the osmotic stress condition. In other words, the positive effect of JA in defending against osmotic stress may be a consequence of its effect of promoting crown root growth.

So, when exposed to different concentrations of PEG, especially under the relatively low osmotic stress condition (≤20% PEG), *cpm2* always loses more growth rate (shoot and root length, shoot and root mass) compared with WT, which indicates that WT exhibited more resistance to weak osmotic stress. Moreover, the greater increase of crown roots in WT suggests that the positive effect of JA in defending against osmotic stress might be correlated with its effect of promoting the growth of crown roots.

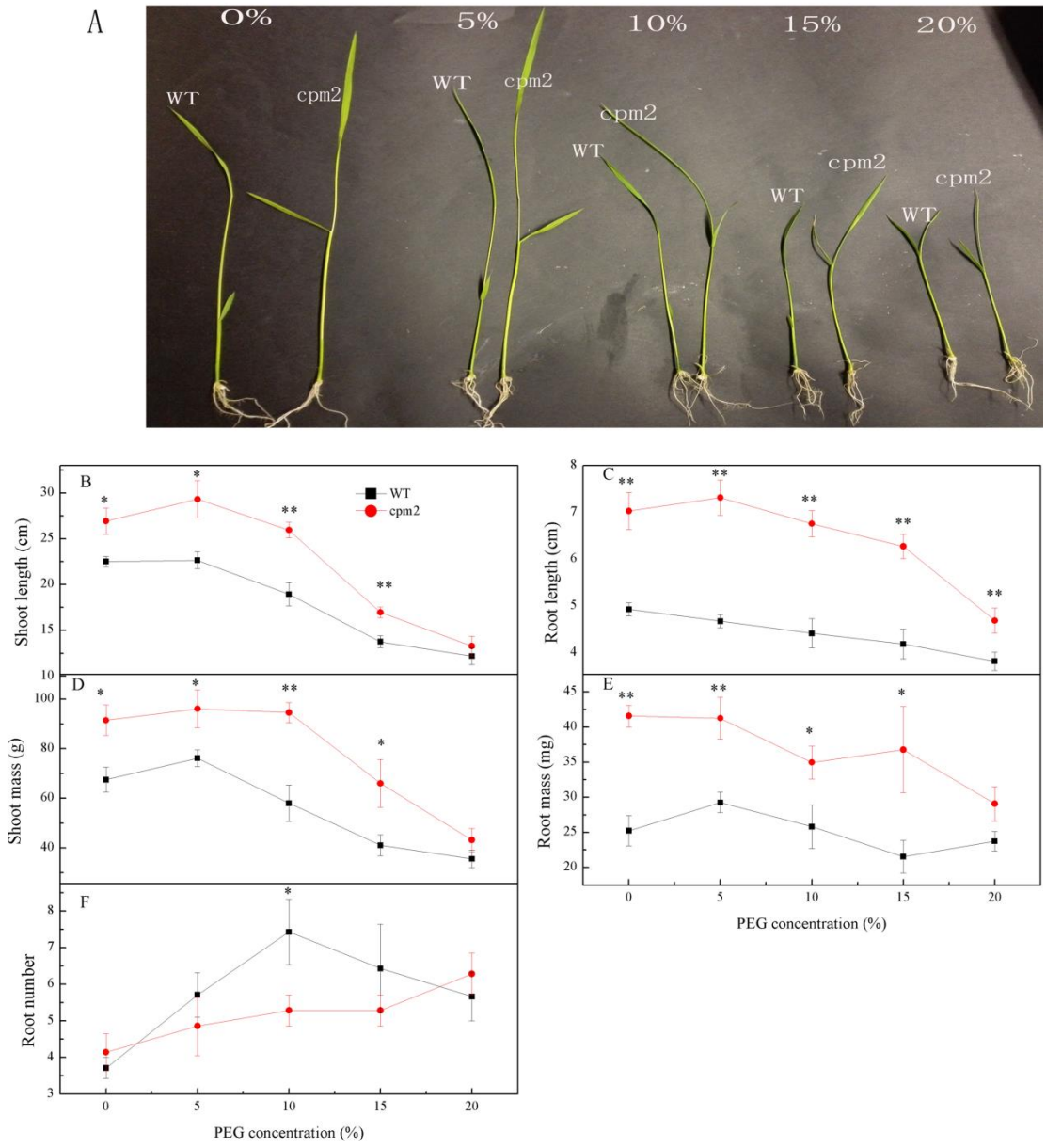

Figure 3.2. Morphology change of wild type (WT) and JA biosynthesis mutants (*cpm2*) when

exposed to different concentrations of PEG 6000 for 3 d. (A) Effects of different concentrations (0%, 5%, 10%, 15%, and 20%) of PEG 6000 on the phenotype of the WT and *cpm2*. (B) Shoot length, (C) root length, (D) shoot mass, (E) root mass, and (F) root number change of WT and *cpm2* after different concentrations (0%, 5%, 10%, 15%, and 20%) of PEG 6000 for 3 d. Here, root number means crown root. Values are means ± SE obtained from six plants. Asterisks indicate statistically significant differences between the WT and *cpm2* in each concentration (0%, 5%, 10%, 15%, and 20%) of PEG treatment (student's t test; *P < 0.05 and **P < 0.01).

3.2 The effect of osmotic stress on hormones of rice shoot and root

As one of the most important signal molecules, hormones play a crucial role in defending against osmotic stress (Fujita et al., 2011). This study aims to elucidate the effect of JA on rice responding to osmotic stress. Therefore, the study of hormones (JA and ABA) constitutes a primary task of the current study. Drought stress usually starts with soil. Specifically, when exposed to drought stress, plant roots may be affected earlier and more directly than shoots, and thus it is necessary to investigate the change of plant hormone in both rice shoots and roots. In our study, we utilized PEG to induce osmotic stress in order to imitate drought stress. Similarly, rice roots contacted with PEG directly. Therefore, in our experiments, plant roots received osmotic stress signals more directly than those of plant shoots. Consequently, in rice roots and shoots, changes of plant hormones would be dissimilar. To thoroughly investigate the responses of multiple plant issue and organs to osmotic stress, we measured changes of hormone content of rice roots and shoots under osmotic stress, respectively. Among them, the intermediate OPDA, JA, and its active material JA-isoleucine (JA-ile) were chosen for the synthesis process. Furthermore, because ABA plays an essential role in plant osmotic stress, changes of ABA content were also measured.

3.2.1 Time course of JAs in response to osmotic stress

In nature, the environment for plants is complex. The challenges that need to be faced and stresses from which they may suffer are also various. In contrast, however, there are few kinds of plant hormones and other signals. Then, how do plants use limited signals to adjust in order to adapt to multiple stresses and complex living environments? Evidence exists suggesting that plants' destiny (survival vs. death) probably depends on the time and spatial location of the appearance and disappearance of signals in cells (Ismail et al., 2014). In other words, even for the same hormone, the time of its appearance and disappearance may determine whether the plant can adapt to stress or die. So, in our experiments, we firstly measured changes of hormone content at six different time points (after PEG treatment). The six time points are untreated (0 h), 0.5 h, 1 h, 3 h, 6 h, and 24 h after PEG treatment. Considering that OPDA and JA can hardly be compounded in *cpm2*, the content will be continually low. Consequently, we only used WT as our material in this experiment.

After PEG treatment to rice, the OPDA content of plant shoots began to increase after 3 h. When it reached 24 h, it still remained at a high level without any indication of decrease (Fig. 3.3A). In roots, however, OPDA only had an apparent increase at 0.5 h, and then started to reduce. At following time points, OPDA content in rice root that was treated by PEG had no changes (compared with control) (Fig. 3.3B). In shoots, JA content increased in 0.5 h after PEG treatment, reached the peak at 1 h, and then began to reduce. Until 24 h after treatment, JA content in PEG treatment was always higher than that in the control (Fig. 3.3C). In roots, JA content only rose apparently 0.5 h after PEG treatment and started to decrease 1 h after treatment (Fig. 3.3D). From 3 h, JA content in PEG treatment was lower than that in the control (Fig. 3.3D). Similarly, JA-Ile in shoots also rose in 0.5 h after PEG treatment, and reached its peak after 1 h. At following time points, although JA-Ile began to reduce, JA-Ile in PEG treatment was always higher than that in the control until 24 h (Fig. 3.3E). In roots, JA-Ile in PEG treatment at 0.5 h and 1 h was obviously higher than that in the control, among which 0.5 h was the peak (Fig. 3.3F). From 3 h to 24 h, JA-Ile in PEG

treatment was lower than that in the control treatment. JA-related hormones in roots changed apparently faster than those in shoots. In addition, the existing time of JA-related hormones in roots was obviously shorter than that in shoots. Usually, 1 h after treatment, JA-related hormones in roots finished the whole process of accumulating, and then decreased to a normal level and even down-regulated compared with the control; whereas, in shoots, the accumulation of JA-related hormones could last for at least 24 h.

According to the results of the time course of JAs, the feature of JA-related hormones in roots was “appear early and also disappear early”; whereas in shoots, JA-related hormones tended to “appear late and disappear late” in response to osmotic stress.

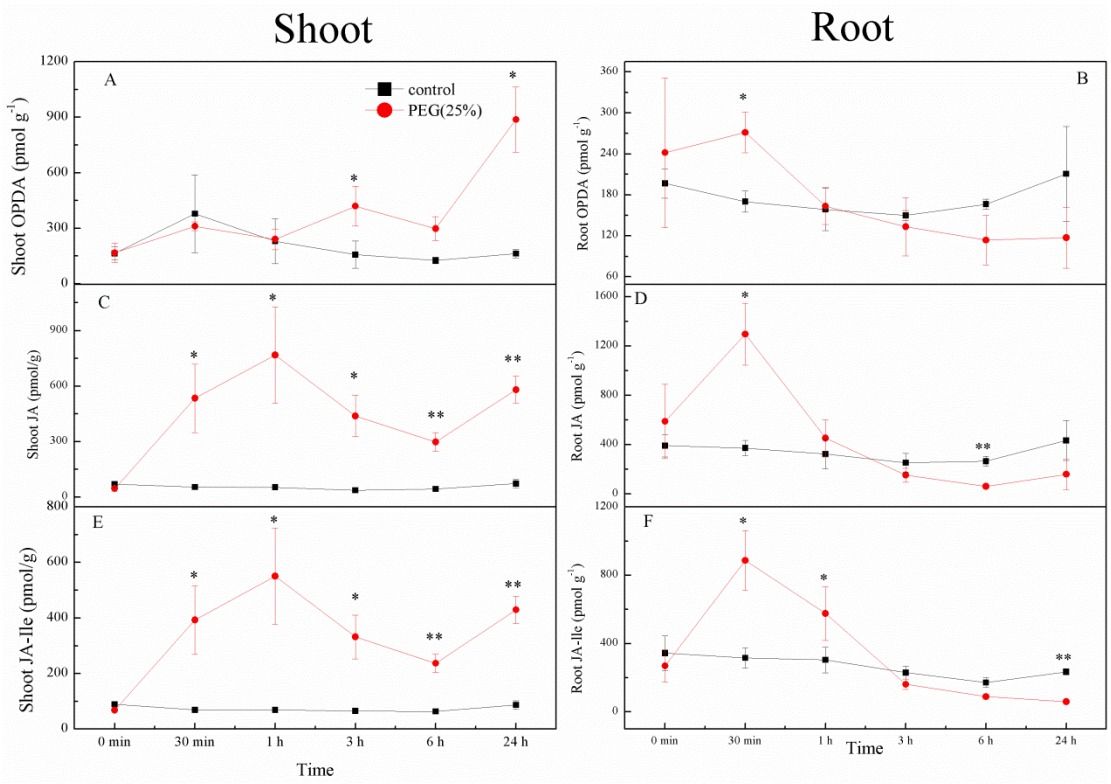

Figure 3.3. Time course of phytohormone levels in rice (WT) shoots and roots in response to osmotic stress (25% PEG 6000). (A) Shoot 12-cis-oxophytodienoic acid (OPDA), (B) root 12-cis-oxophytodienoic acid (OPDA), (C) shoot jasmonic acid (JA), (D) root jasmonic acid (JA), (E) shoot JA-isoleucine (JA-Ile), and (F) root JA-isoleucine (JA-Ile) in response to osmotic stress at different time points (0, 0.5, 1, 3, 6, and 24 h). Control plants were incubated in H_2O for the

times indicated, respectively. Values represent the mean of three independent experiments ± SE. Means followed by asterisks indicate statistically significant differences between the control and PEG 6000 treatment in each time point (student's t test; *P < 0.05 and **P < 0.01).

3.2.2 Time course of ABA in response to osmotic stress

Compared with JA, the accumulation speed of ABA in rice is apparently slower, both in rice shoots and roots (Fig. 3.4A). In rice shoots, ABA rose 1 h after treatment, reached its peak after 6 h, and then decreased after 24 h of PEG treatment. However, until the end of the experiment, ABA content of rice shoots being treated with PEG was still higher than that without treatment (control) (Fig. 3.4A). In shoots, ABA content after 3-h- and 6-h-treatment could almost reach 3000 pmol/g, which is much higher than that in the control. In roots, ABA content could only reach approximately 700 pmol/g after PEG treatment (Fig. 3.4A-B). In rice roots, ABA content began to increase 1 h after PEG treatment, reached the peak after 3 h (Fig. 3.4B), and then began to decrease. Until 24 h after treatment, ABA content of rice roots in PEG was still higher than that in the control (Fig. 3.4B), which is different from JAs.

According to the results of the time course of ABA, the feature of ABA in roots was also "appear early and also disappear early"; and in shoot, ABA the trend was also "appear late and disappear late" in response to osmotic stress, which is somewhat similar to JAs. However, we also found that, compared with JA, the change (increase and decrease) of ABA was always slightly later than JAs. In addition, the accumulation of ABA in shoots was much higher than that in roots (3000p mol/g vs. 700 pmol/g), which may suggest that the function of ABA in shoots was more critical in response to osmotic stress.

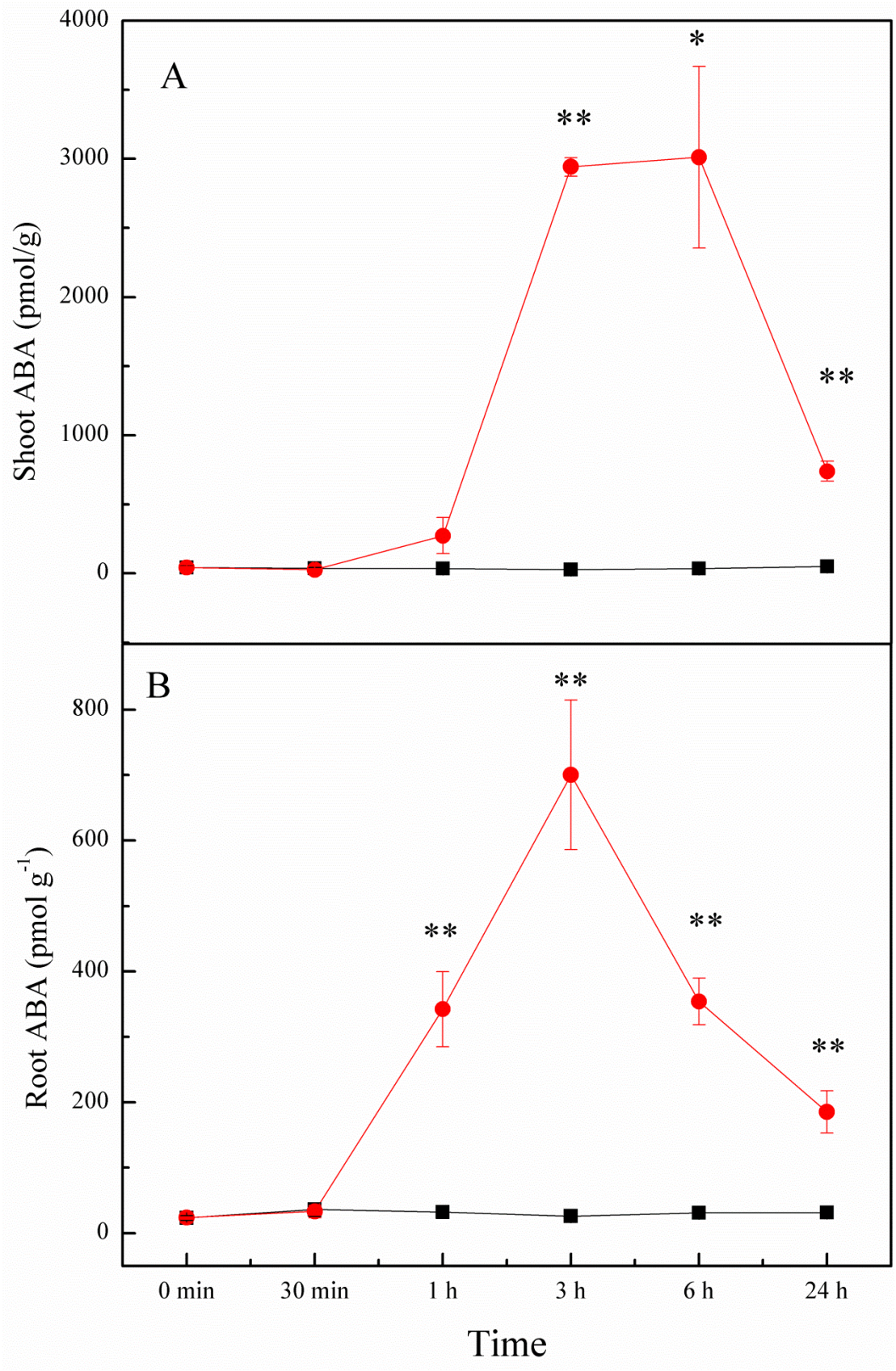

Figure 3.4. Time course of ABA levels in rice (WT) shoots and roots in response to osmotic stress (25% PEG 6000). (A) Shoot abscisic acid (ABA) and (B) root abscisic acid (ABA) in response to

osmotic stress at different time points (0, 0.5, 1, 3, 6, and 24 h). Control plants were incubated in H_2O for the times indicated, respectively. Values represent the mean of three independent experiments ± SE. Means followed by asterisks indicate statistically significant differences between the control and PEG 6000 treatment in each time point (student's t test; *P < 0.05 and **P < 0.01).

3.2.3 JA was required for physiological adaption to osmotic stress

Hormones responding to abiotic stresses is usually a rapid process. So, when plants suffer from abiotic stresses, changes of hormones will appear in a short period of time. JA-related hormones can hardly accumulate in *cpm2*. Therefore, in the time course experiment which requires a substantial amount of experimental materials, we did not use *cpm2*. Since the synthesis of ABA is unaffected by AOC deletion, it is still necessary to study changes of plant hormones in *cpm2* in response to osmotic stress. From the experiments above, ABA accumulated greatly in 3 h after being treated with PEG. Consequently, we chose 3 h after PEG treatment as the time point for the measurement. Changes of hormones were fairly similar to what we anticipated. We could observe that either in *cpm2* roots or shoots, OPDA, JA, and JA-ile content was extremely low. Indeed, they were hardly effected by osmotic stress (Fig. 3.5A-F). However in WT shoots, compared with the control, either OPDA, JA, or JA-ile content rose apparently after plants suffered strong osmotic stress (25% PEG), and the difference between the control reached an extremely significant level ($p<0.01$) (Fig. 3.5A, C, E). In roots, however, no difference was found between OPDA, JA, and JA-ile (Fig. 3.5B, D, F). It is not clear why these hormones had such a large difference in shoots, but not in roots. It is most likely that, when it was at the 3-h time point, hormones in WT roots had already reduced from a peak to a lower level, and at this time point there were no large differences (compared with the control).

The result of the present work suggested that JA was required for adaption to osmotic stress, as the increase of JA in WT helped rice to better adapt to osmotic stress.

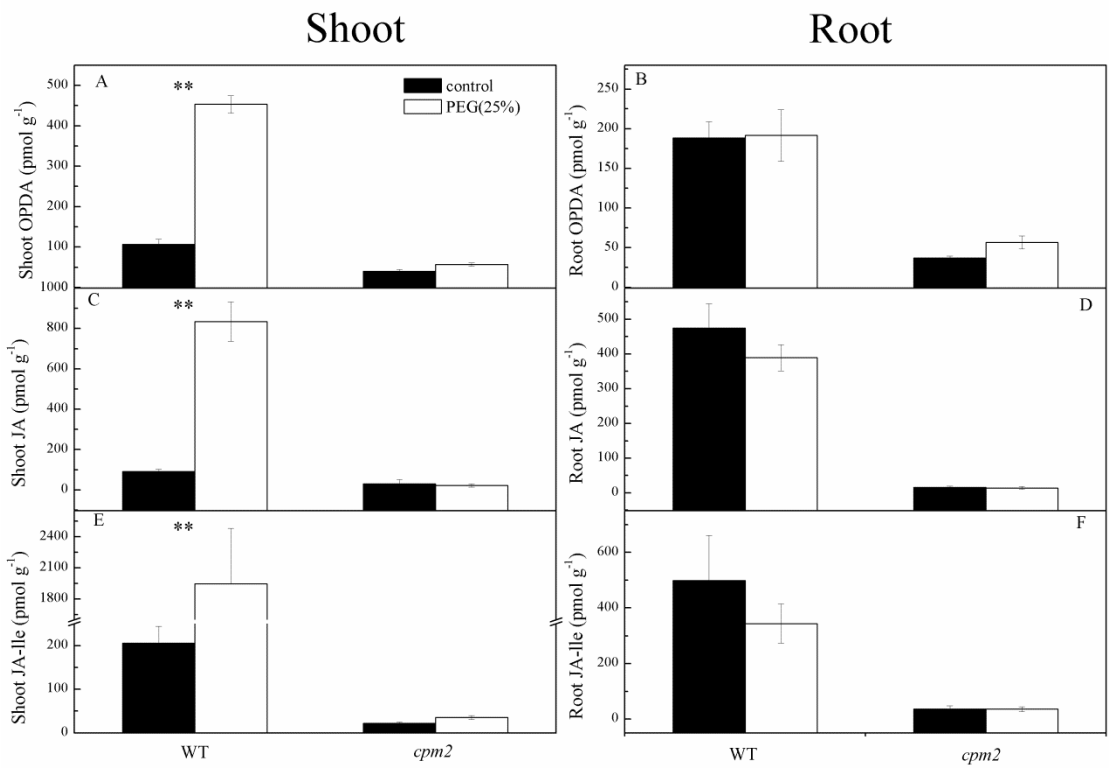

Figure 3.5. Level of phytohormones in shoots and roots of wild type (WT) and JA biosynthesis mutants (*cpm2*) under control conditions and 3 h of osmotic stress (25% PEG 6000). (A) Shoot 12-cis-oxophytodienoic acid (OPDA), (B) root 12-cis-oxophytodienoic acid (OPDA), (C) shoot jasmonic acid (JA), (D) root jasmonic acid (JA), (E) shoot JA-isoleucine (JA-Ile), and (F) root JA-isoleucine (JA-Ile) in response to osmotic stress at different time points. Values represent the mean of at least four independent experiments ± SE. Results for the control and treatment are indicated by black and white bars, respectively. Significant differences among different treatments or genotypes are indicated by asterisks, according to Tukey's Honestly Significant Difference (HSD) test (*P < 0.05 and **P < 0.01).

3.2.4 ABA was increased in response to osmotic stress

Different from JA, ABA content increases to a certain degree under osmotic stress in any genotype (Fig. 3.6). Since the synthesis pathways of ABA are not damaged in *cpm2*, its synthesis is also not blocked. Under the normal condition, ABA content in rice shoot and roots is approximately 40 pmol g^{-1}. Under osmotic stress, however, ABA content in shoots can reach 2000～5000 pmol g^{-1}. In roots, it can only rise to

500～600 pmol g^{-1} generally. In other words, the changes of ABA content in shoots are much more than those in roots. Contrary to our expectations, the accumulation of ABA content in WT shoots is much higher than that of *cpm2* under the osmotic stress condition (Fig 3.6 A); whereas, in roots, the accumulation of ABA content in WT is lower than that in *cpm2* (Fig 3.6 A) when it experienced osmotic stress.

The strong increase of ABA under the osmotic stress condition (in both WT and *cpm2*) indicated that ABA might be required for adaption to osmotic stresses. In addition, ABA was induced significantly in the absence of JA, which suggested that ABA and JA pathways respond to osmotic stress in parallel. However, in rice, both hormonal pathways seem to be induced by osmotic stress more or less independently, as jasmonates were induced earlier than ABA and we found that *cpm2* mutants accumulated less ABA in shoots than the WT. Thus, JAs might support plants to accumulate higher levels of ABA in shoots.

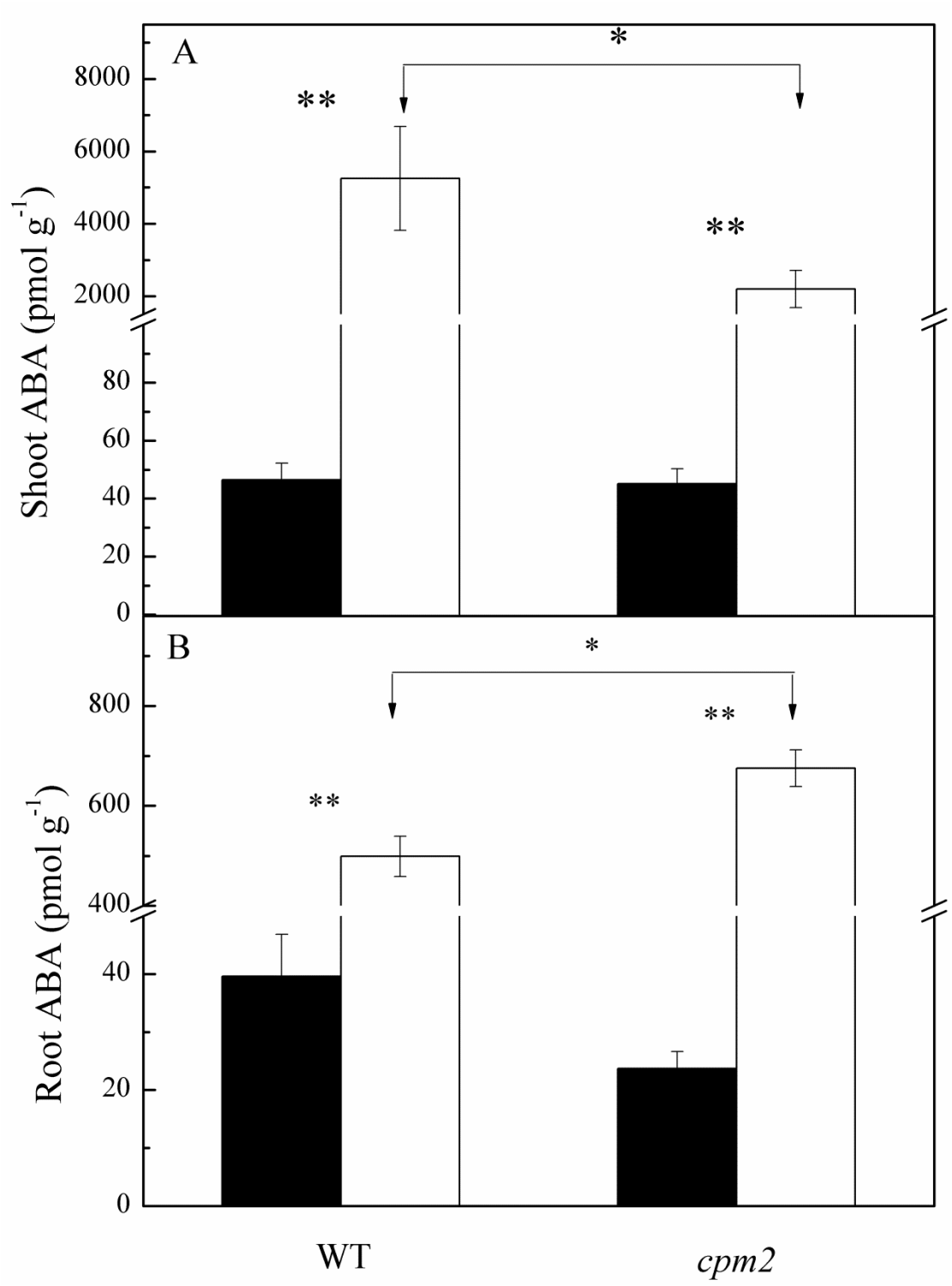

Figure 3.6. The level of ABA in shoots and roots of wild type (WT) and JA biosynthesis mutants (*cpm2*) under control conditions and 3 h of osmotic stress (25% PEG 6000). (A) Shoot abscisic acid (ABA) and (B) root abscisic acid (ABA) in response to osmotic stress at different time points. Values represent the mean of at least four independent experiments ± SE. Results for the control and treatment are indicated by black and white bars, respectively. Significant differences amongst different treatments or genotypes are indicated by asterisks, according to Tukey's Honestly

Significant Difference (HSD) test (*P < 0.05 and **P < 0.01).

3.3 Gene response to osmotic stress

Under drought conditions, the content of endogenous hormones (e.g., ABA) rises and activates encoded transcription factors, metabolism enzymes, and target genes for other substances located at downstream positions (Yamaguchi-Shinozaki and Shinozaki, 2006). By transcription and translation of coding genes downstream, hormones finally become proteins that have effects on plants' defense against adversity stress. So, besides changes of hormones, the study of gene expression also constitutes a key point of the current study. In our study, the expression level of gene in WT at 1 h (control treatment) is used as a reference system (relative expression = 1).

3.3.1 Osmotic stress can induce the expression of JA synthetic genes upstream of AOC. This phenomenon is more obvious in WT.

Synthesis of JA originates from α-linolenic acid, in which OPDA is firstly formed through a series of reactions. Among them, AOS and AOC are the necessary enzymes in this synthesis process, which are located upstream of our mutant (AOC). In the present study, we chose two time points after PEG treatment, 1 h and 3 h, as we determined that gene expression in the first 3 h (after PEG treatment) was obvious. The expression level of the gene of *cpm2* almost remained the same after PEG treatment. Except in the 1 h time point, *OsAOS1* gene in *cpm2* roots was slightly increased after PEG treatment (Fig. 3.7B). However, in WT, these genes all seemed to change greatly. In WT shoots, 1 h and 3 h after PEG treatment, *OsAOS1*, *OsAOS2*, and *OsAOC* upregulated significantly (Fig. 3.7A, C, E). The upregulation amplitude in 1 h seemed to be higher than that in 3 h, which indicated that these three genes might be short-time genes in response to strong osmotic stress. In roots, we found the upregulation of gene (*OsAOS1*, *OsAOS2*, *OsAOC*) in only 1 h after PEG treatment, which was not found at the 3 h time point (Fig. 3.7B, D, F). This suggested that the

speed of gene expression in roots was faster than that in shoots, which is in accordance with the result of hormone accumulation (Fig. 3.3A-F).

The result of the study suggested that JA synthetic genes (*OsAOS1*, *OsAOS2*, and *OsAOC*), which located upstream of AOC, were upregulated in WT, but not in *cpm2*. Moreover, all of these genes are short-time response genes.

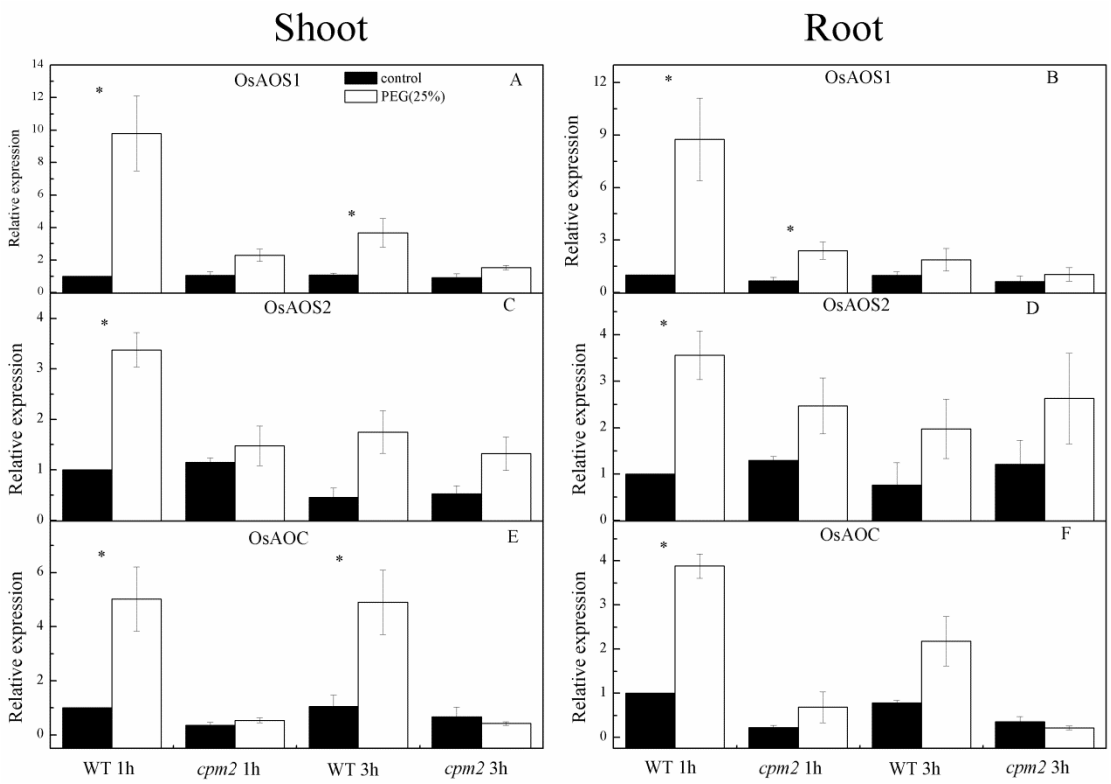

Figure 3.7. Transcriptional regulation of selected JA biosynthesis genes in response to osmotic stress (25% PEG 6000) in shoots and roots of wild type (WT) and JA biosynthesis mutants (*cpm2)*. WT and *cpm2* have been exposed to control (water, black bars) and osmosis stress (25% PEG 6000) for 1 h and 3 h. The transcription levels of the genes (A) shoot *OsAOS1*, (B) root *OsAOS1*, (C) shoot *OsAOS2*, (D) root *OsAOS2*, (E) shoot *OsAOC*, and (F) root *OsAOC* were quantified relative to control plants after normalization with housekeeping gene *OsUBQ5*. Values represent the mean of three independent experiments ± SE. Significant differences among different treatments or genotypes at two time points (1 h and 3 h) are indicated by asterisks, according to Tukey's Honestly Significant Difference (HSD) test (*P < 0.05 and **P < 0.01).

3.3.2 Osmotic stress can only slightly induce the gene expression of

JA synthetic genes downstream of AOC

OPDA is first formed from α-linolenic acid by a series of reactions. Then, JA is finally formed also by a series of reactions. Among them, the expression of AOC and JAR plays an important role in the synthesis of JA. OPR7 and JAR1 are also JA synthetic genes. Since they are located downstream of our mutant AOC, we term them *synthetic genes downstream of AOC*. Different from the genes upstream of AOC, the upregulation of these two genes is not obvious. *OsJAR1* has no changes in any genotype (Fig. 3.8C, D) in response to osmotic stress. The same as the genes upstream of AOC, the expression level of genes is extremely low in *cpm2*, regardless of whether or not it was treated with PEG. In WT, however, 1 h after PEG treatment, there is still slight upregulation of *OsOPR7* gene.

Compared with the JA synthetic genes that are located upstream of AOC, the genes downstream of AOC exhibited a much lower upregulation. Only *OsOPR7* was slightly upregulated after 1 h of PEG treatment.

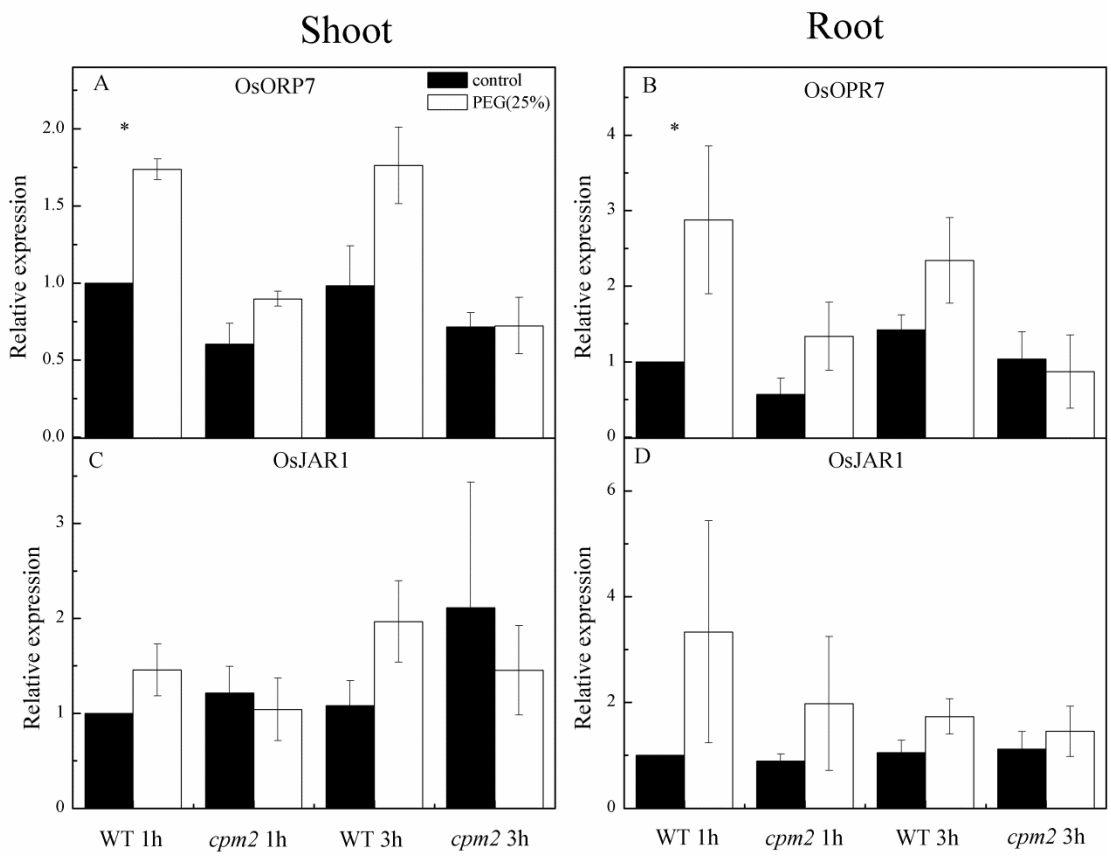

Figure 3.8. Transcriptional regulation of selected JA biosynthesis genes in response to osmotic stress (25% PEG 6000) in shoots and roots of wild type (WT) and JA biosynthesis mutants (*cpm2)*. WT and *cpm2* have been exposed to control (water, black bars) and osmosis stress (25% PEG 6000) for 1 h and 3 h. The transcription levels of the genes (A) shoot *OsOPR7*, (B) root *OsOPR7*, (C) shoot *OsJAR1*, and (D) root *OsJAR1* were quantified relative to control plants after normalization with housekeeping gene *OsUBQ5*. Values represent the mean of three independent experiments ± SE. Significant differences among different treatments or genotypes at two time points (1 h and 3 h) are indicated by asterisks, according to Tukey's Honestly Significant Difference (HSD) test (*P < 0.05 and **P < 0.01).

3.3.3 JA response gene (JAZ) is upregulated in WT, but not in *cpm2* after PEG treatment.

In the present study, the expression of JAZ gene (*OsJAZ1*, *OsJAZ4*, *OsJAZ11*) in *cpm2* was found to be extremely low whether or not it was treated with PEG. In other words, JAZ genes did not respond to osmotic stress in *cpm2*. However, in WT, there was an obvious upregulation after PEG treatment in both roots and shoots (Fig. 3.9 A-D). In shoots, 1 h after WT suffering from osmotic stress, *OsJAZ4* gene upregulated approximately 2.5 times (Fig. 3.9 A); whereas, in roots, *OsJAZ1* gene upregulated approximately 3.5 times after PEG treatment (Fig. 3.9 B), and there was no upregulation 3 h after PEG treatment (Fig. 3.9 A-B). This could also suggest that *OsJAZ1* and *OsJAZ4* genes were probably two short-time genes, as well. In 1 h and 3 h after PEG treatment, *OsJAZ11* gene in WT shoots upregulated approximately 7.5 times and 9 times, respectively (Fig. 3.9 C). In roots, it upregulated approximately 5 times and 6 times, respectively (Fig. 3.9 D).

The result of the present study indicated that JA response genes (mainly JAZ genes) are upregulated in WT, but not in JA deficiency mutant (*cpm2*). This result agrees well with the change of JAs.

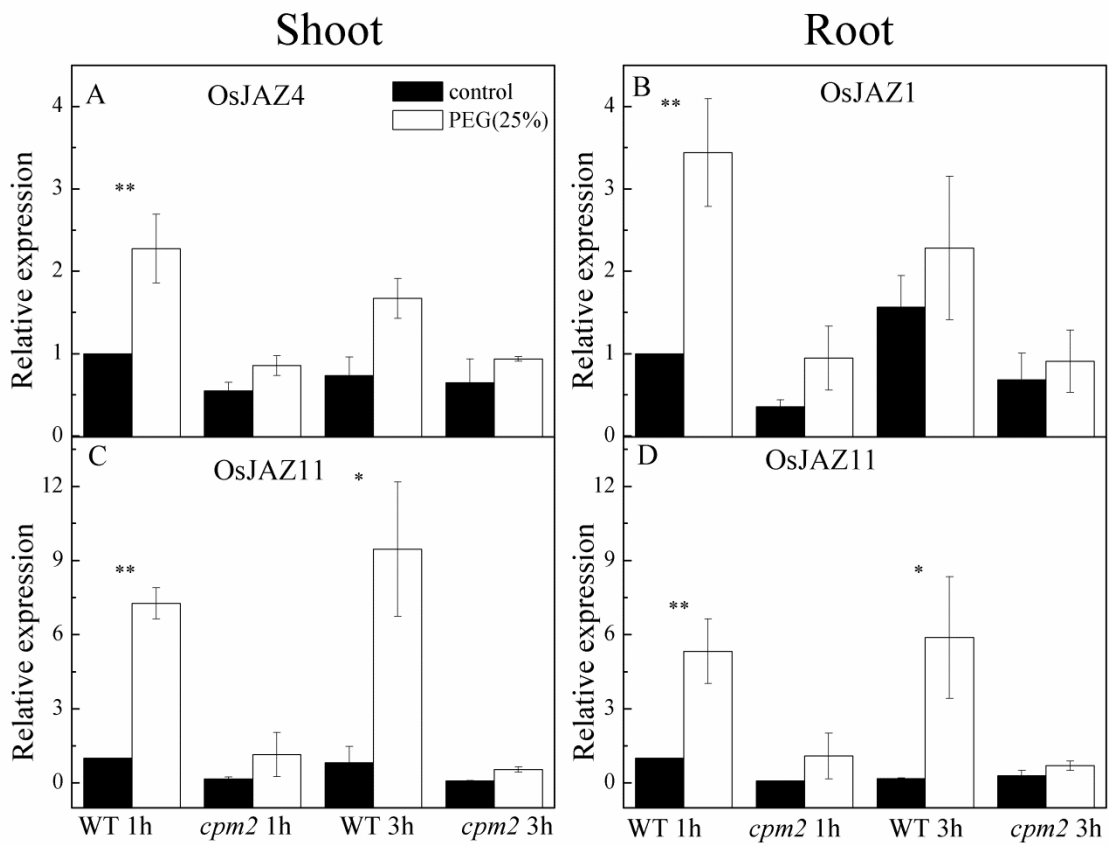

Figure 3.9. Transcriptional regulation of selected JA signaling genes in response to osmotic stress (25% PEG 6000) in shoots and roots of wild type (WT) and JA biosynthesis mutants (*cpm2*). WT and *cpm2* have been exposed to control (water, black bars) and osmosis stress (25% PEG 6000) for 1 h and 3 h. The transcription levels of the genes (A) shoot *OsJAZ4*, (B) root *OsJAZ1*, (C) shoot *OsJAZ11*, and (D) root *OsJAZ11* were quantified relative to control plants after normalization with housekeeping gene *OsUBQ5*. Values represent the mean of three independent experiments ± SE. Significant differences among different treatments or genotypes at two time points (1 h and 3 h) are indicated by asterisks, according to Tukey's Honestly Significant Difference (HSD) test (*P < 0.05 and **P < 0.01).

3.3.4 ABA-related genes upregulated more in WT shoots compared with *cpm2* shoots. In roots, *OsLEA3* expressed more in *cpm2*.

LEA protein was first characterized in cottonseed during the maturation phase of embryogenesis approximately 30 years ago (Liu et al., 2010). After this, many LEA proteins were found to accumulate in plant vegetative organs when facing various

stresses, such as cold, drought, and salt stress (Battaglia et al., 2008; Bies-Etheve et al., 2008). According to its expression pattern and sequence, LEA proteins can be divided into 5-9 groups (Liu et al., 2010). One group is characterized by a repeated 11-mer amino acid motif (TAQAAKEKAGE) (Dure III, 1993). Although LEA3 gene is not the response gene of ABA, numerous recent studies demonstrated that LEA3 gene usually upregulated greatly when ABA accumulated (Lu et al., 2009; Kim et al., 2011; Feng et al., 2014). So, in our study, we used LEA3 as the marker gene of ABA. Different from the JA-related genes above, *OsLEA3* gene was upregulated in both WT and *cpm2* when exposed to osmotic stress. In shoots, 1 h after PEG treatment, *OsLEA3* gene of WT upregulated approximately 25 times, while *cpm2* upregulated approximately 10 times (Fig. 3.10 A). After 3 h, it upregulated approximately 250 times in WT, while approximately 180 times in *cpm2* (Fig. 3.10A). In roots, 1 h after PEG treatment, *OsLEA3* upregulated approximately 30 times in both WT and *cpm2*; whereas, 3 h after treatment, *OsLEA3* gene upregulated approximately 300 times in WT and approximately 500 times in *cpm2* (Fig. 3.10B). In shoots, the upregulation amplitude of *OsLEA3* in WT was significantly higher than that of *cpm2*. In roots, the upregulation amplitude of *OsLEA3* in *cpm2* was higher than that of WT, which we cannot yet precisely explain. In addition, expression of *OsLEA3* gene was still very high after 3 h of PEG treatment. This indicated that, compared with the JA-related genes that we checked above, upregulation of *OsLEA3* gene was longer (1 h vs. 3 h), which was in accordance with the result of ABA in rice having longer accumulation time than JA.

The result revealed that *OsLEA3* was strongly induced under the osmotic stress condition. Moreover, in shoots, *OsLEA3* expressed more in WT; whereas, in roots, the opposite was the case. The upregulation of *OsLEA3* gene sustained for a longer time compared to JA-related genes. This provided further evidence that, in rice, both hormonal (JA and ABA) pathways seem to be induced by osmotic stress more or less independently. It is also found that JAs might support plants to accumulate higher levels of ABA in shoots.

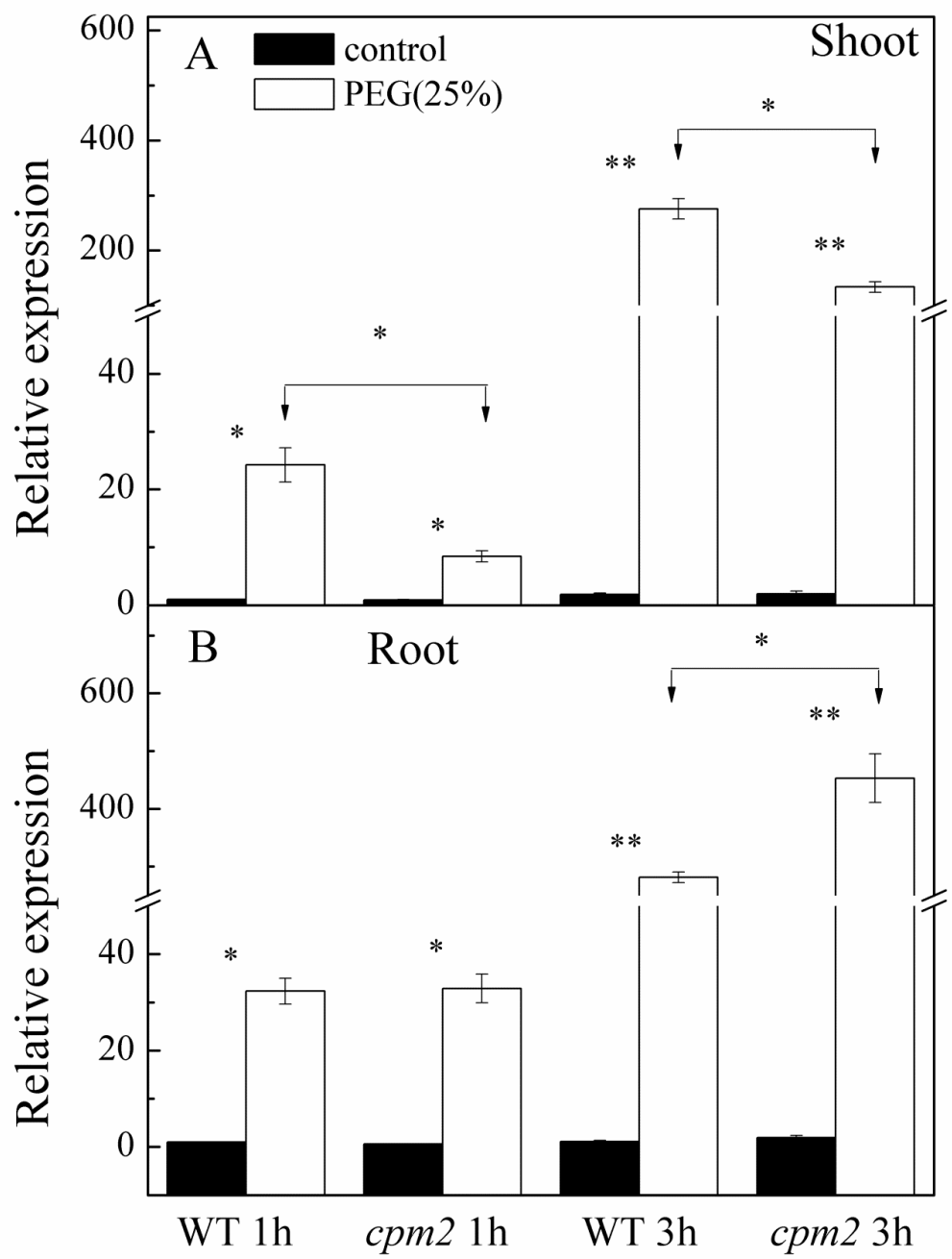

Figure 3.10. Transcriptional regulation of *OsLEA3* in response to osmotic stress (25% PEG 6000) in shoots and roots of wild type (WT) and JA biosynthesis mutants (*cpm2*). WT and *cpm2* have been exposed to control (water, black bars) and osmosis stress (25% PEG 6000) for 1 h and 3 h. The transcription levels of the genes (A) shoot *OsLEA3* and (B) root *OsLEA3* were quantified relative to control plants after normalization with housekeeping gene *OsUBQ5*. Values represent

the mean of three independent experiments ± SE. Significant differences among different treatments or genotypes at two time points (1 h and 3 h) are indicated by asterisks, according to Tukey's Honestly Significant Difference (HSD) test (*P < 0.05 and **P < 0.01).

3.4 Osmotic stress can induce the decline of stomatal conductance in rice. WT showed more downregulation compared to *cpm2*.

From the experiments above, ABA and JA content would increase greatly when rice suffered from osmotic stress. We also know that ABA has a close relation with stomatal conductance (Gs). Usually, the increase of ABA can directly lead to stomatal closure and the decline of Gs (Huntingford et al., 2015; Negin and Moshelion, 2016). Of course, previous studies also suggested that the increase of JA can directly result in stomatal closure and the decline of Gs (Suhita et al., 2004). In addition, opening and closing of stomata is critical to plant water exchange between leaves and the atmosphere (Xu and Zhou, 2008), especially under drought stress (Resco de Dios et al., 2016). In our study, 3 h after suffering from osmotic stress, Gs of rice had an obvious decrease in two genotypes (Fig. 3.11). In WT, Gs reduced from approximately 250 mmol m^{-2} s^{-1} to 75 mmol m^{-2} s^{-1}; whereas, in *cpm2,* Gs reduced just from approximately 250 mmol m^{-2} s^{-1} to 150 mmol m^{-2} s^{-1} (Fig. 3.11). So, the decrease degree of Gs in WT was much more obvious than that in *cpm2*. This result is in accordance with our results that ABA and JA increased more in WT compared to *cpm2* when treated with PEG. According to our result, it can be concluded that, compared with *cpm2*, stomatal conductance in WT was more sensitive to osmotic stress.

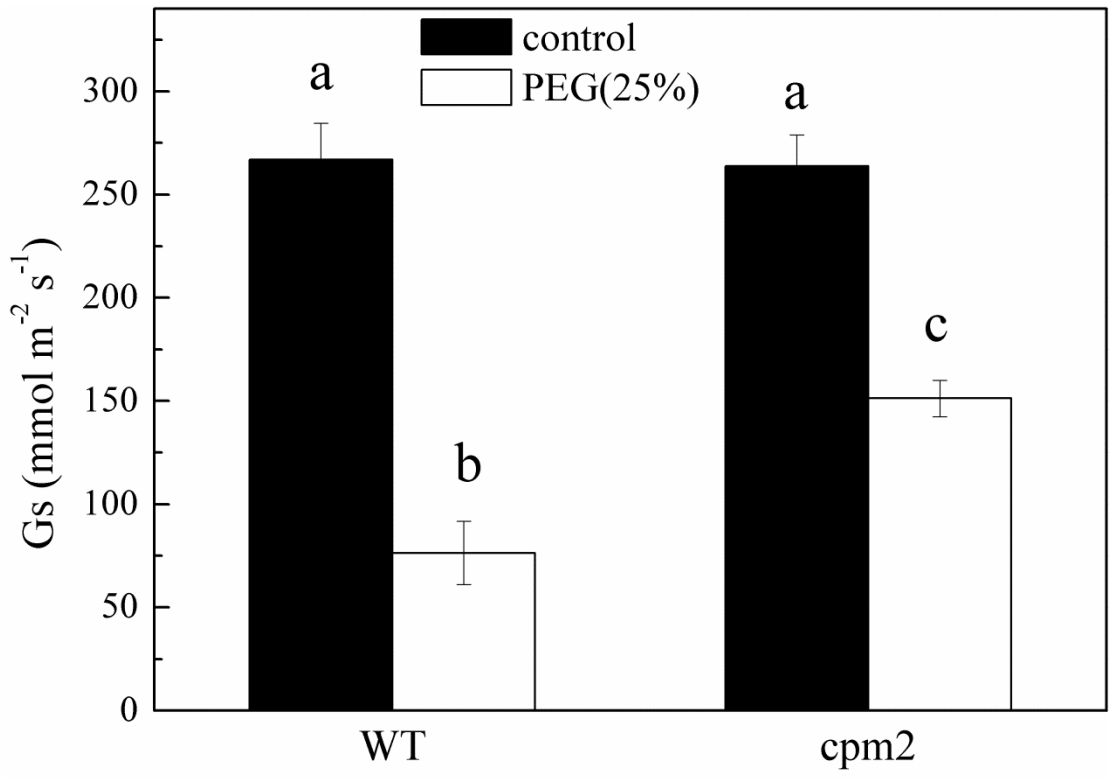

Figure 3.11. Effect of osmotic stress (25% PEG 6000) on stomatal conductance (Gs) in wild type (WT) and JA biosynthesis mutants (*cpm2*). Three-week-old rice WT and *cpm2* were used for the measurement of Gs in control (water) and osmotic stress condition (25% PEG 6000) for 3 h. Values are means ± SE of eight plants. Asterisks indicate statistically significant differences between the control and 25% PEG 6000 treatment in two genotyped (WT and *cpm2*) (student's t test; *P < 0.05 and **P < 0.01).

3.5 JA biosynthesis mutant (*cpm2*) accumulated more ROS compared to WT.

Reactive oxygen species (ROS) is a kind of by-product of aerobic cells in aerobic metabolism processes, including O_2^-, peroxide, and oxygen free radicals (Turrens, 2003; Apel and Hirt, 2004). ROS is also an essential index. On the one hand, plants will produce many ROS under adversity stress, which usually has a great effect on damaging plant cells, especially membrane lipids and genes, and has a severe influence on different kinds of chemical reactions in plant cells (Yamamoto et al., 2002). On the other hand, ROS (especially in low concentrations) can affect a series

of signal transduction pathways (Mittler et al., 2011). In our study, we chose O_2^- and MDA as our research index. Because MAD is normally the product of cell membrane lipid peroxidation, and its accumulation in plants is always resultant from the accumulation of peroxide, we also treated MAD as ROS in our study (Hazman et al., 2015). In the present study, there were a few changes of O_2^- in WT shoots 24 h after PEG treatment. However, there was a large quantity of accumulation of O_2^- in *cpm2* (Fig. 3.12A). In roots, we could not find any obvious change of O_2^- in WT or *cpm2* after PEG treatment (Fig. 3.12B). In addition, *cpm2* accumulated more MDA compared with WT in both rice shoots and roots (Fig. 3.12C-D). As the content of O_2^- and MDA was somewhat high after PEG treatment, in all probability, the ROS in our study is a toxic substance that can induce damage. This result is also in accordance with the result that *cpm2* exhibited more sensitivity to osmotic stress.

The result of the present study indicated that, compared with WT, *cpm2* accumulated more ROS when exposed to osmotic stress. Thus, JA may be correlated with the accumulation of ROS in response to osmotic stress, and this correlation is negative. However, precisely how JA inhibits ROS accumulation remains unclear. It may be due to that JA correlated with ROS synthesis (negative correlation) or the ROS elimination system (positive correlation).

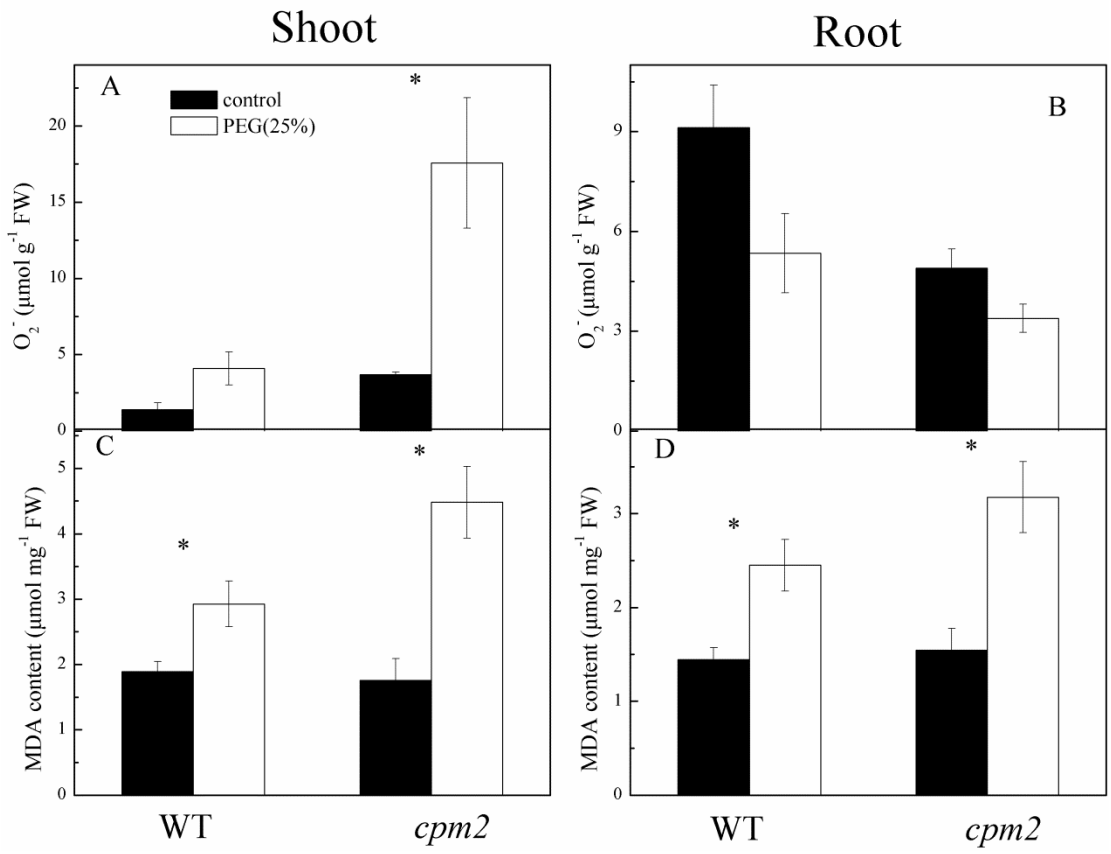

Figure 3.12. Lipid peroxidation and superoxide levels in wild type (WT) and JA biosynthesis mutants (*cpm2*) shoots and roots under osmotic stress condition (25% PEG 6000) for 24 h. (A) Levels of superoxide in the WT and *cpm2* in shoots of control and osmotic-stressed plants. (B) Levels of superoxide in the WT and *cpm2* in roots of control and osmotic-stressed plants. (C) The level of malondialdehyde (MDA) was estimated in the WT and *cpm2* in shoots of control and osmotic-stressed plants. (D) The level of malondialdehyde (MDA) was estimated in the WT and *cpm2* in roots of control and osmotic-stressed plants. Values represent the mean of at least three independent experiments ± SE. Asterisks indicate statistically significant differences between the control and 25% PEG 6000 treatment in two genotypes (WT and *cpm2*) (student's t test; *P < 0.05 and **P < 0.01).

3.6 Changes of metabolites accumulation in rice shoots and leaves under osmotic stress

Amino acids are one of numerous bioactive macromolecules of biological organisms, and the basic material that constructs the cell and restores organization (Maeda and

Dudareva, 2012). The functions of many amino acids have yet not been totally confirmed (Li et al., 2007). In our study, we used gas chromatography mass spectrometry (GCMS) to measure more than 100 kinds of amino acids and compared their relative changes 6 h and 12 h after PEG treatment. We chose WT and *cpm2* content before treatment as the reference system. Since the amount of rice samples that is needed in this experiment is huge, we only measured amino acids in rice shoots, and not in rice roots. We obtained many results, and only chose six representative amino acids in the results part. Other results are found in the Appendix. The experiment set three biological replicates. Their data were combined due to calculation reasons, and the final results showed no error bars.

Of the currently known amino acids, proline (Pro) seems to be most related to osmotic stress defense (Watanabe et al., 2000). As a small molecular substance, Pro plays an important role in controlling plant osmotic pressure, and maintaining the balance of osmotic pressure between cells and the outside environment (Molinari et al., 2004). In our studies, Pro was accumulated both in WT and in *cpm2* after PEG treatment, while the degree of accumulation in WT was much greater than that in *cpm2* (Fig. 3.13A). Even in the control, the proline content of WT was higher than that of *cpm2* (Fig. 3.13A). On the one hand, this partially explained why WT had stronger resistance compared with *cpm2*. On the other hand, it also suggested that JA might be correlated with proline accumulation in rice. Consequently, in order to further understand the relationship between JA and Pro under osmotic stress, we also chose *OsP5CS*, which is one of the most critical marker genes of Pro for molecular biology work. In our study, we found that the expression level of *OsP5CS* gene greatly increased 12 h after treatment, and the expression in WT was much more than that in *cpm2* (Fig. S6). Since we already confirmed that the upregulation of proline was much greater in WT, the expression levels of *OsP5CS* gene met our expectations.

According to the results, the amino acids can be divided into four groups. The first and most important group is those that can both accumulate in WT and *cpm2* after

PEG treatment. Obviously, WT accumulated much more compared with *cpm2*, which indicated that JA may be correlated with the synthesis of amino acids under the osmotic stress condition. Among them, Pro may be the most typical example. Many other amino acids belong to this group: pyroglutamic acid (Fig. 3.13B), serotonin (Fig. 3.13D), asparagine, allantoin, β-cyanoalanine and coumaroylquinic acid (Fig. S1A, B, D, E), gallic acid, γ-tocopherol, glutamine (Fig. S2B-D), glyceric acid, glycine, homoglutamic acid, isoleucine, lysine (Fig. S3A-C, E-F), methionine, phosphorylethanolamine, phenylalanine (Fig. S4A, D, F), tryptophan, tyramine, tyrosine, valine, and xylose (Fig. S5A-E).

Besides *OsP5CS*, we also chose tryptophan decarboxylase (TDC) gene, which is strongly related with tryptophan and phenylalanine ammonia-lyase (PAL), and related with phenylalanine as marker genes. We treated rice with PEG, and performed semi-qPCR experiments on *OsPAL4*, *OsTDC1*, and *OsTDC2* at the time point of 6 h and 12 h to ensure the expression level at these times. We observed that the expression level of *OsPAL4* gene showed an obvious difference at 12 h, and the upregulation of *OsPAL4* under PEG treatment was obvious in WT. In *cpm2*, whether or not the rice was treated with PEG, the expression level of *OsPAL4* was very low (Fig. S6). In addition, for *OsTDC1* gene, 6 h after PEG treatment, there was an obvious upregulation not only in WT, but also in *cpm2*. Of course, the expression level in WT was also much higher than that in *cpm2* (Fig. S6). Changes of *OsTDC2* appeared at 12 h after PEG treatment. The expression level in WT was again much higher than that in *cpm2* (Fig. S6). Since we already determined that the upregulation of phenylalanine and tryptophan in WT was much greater than that in *cpm2*, the expression levels of these genes met our expectations.

The second group is those that can accumulate both in WT and *cpm2* after PEG treatment. The increasing degree seems equal in both WT and *cpm2*, which indicated that these amino acids might be involved in osmotic stress adaptation in rice. JA, however, seems to have nothing to do with this pathway. This group includes several

amino acids: sorbitol (Fig. 3.13C), β-Alanine, feruloylquinic acid (Fig. S1C, F), and ferulic acid (Fig. S2A).

The third group is those that can accumulate both in WT and *cpm2* after PEG treatment. However, it seems that *cpm2* accumulated more compared with WT. So far, we still cannot explain why these amino acids accumulated more in *cpm2* than in WT. It is possible that they are just the product of damage, as *cpm2* showed more damage compared with WT. Several amino acids belong to this group: sucrose, quinic acid (Fig. 3.13E-F), gallic acid, galactinol (Fig. S2B, E), malic acid, and phosphate (Fig. S3B-C).

The fourth group is those that cannot accumulate in both WT and *cpm2* after PEG treatment, or sometimes even exhibit a decline after PEG treatment. These amino acids have nothing to do with resistance to osmotic stress. Only very few amino acids belong to this group: galacturonic acid (Fig. S2F) and 3-phosphoglyceric acid (Fig. S4E).

According to our results, most amino acids accumulated more in WT than in *cpm2*. This may indicate that the accumulation of these amino acids was correlated with JA, and they may assist plants to have more resistance in response to osmotic stress.

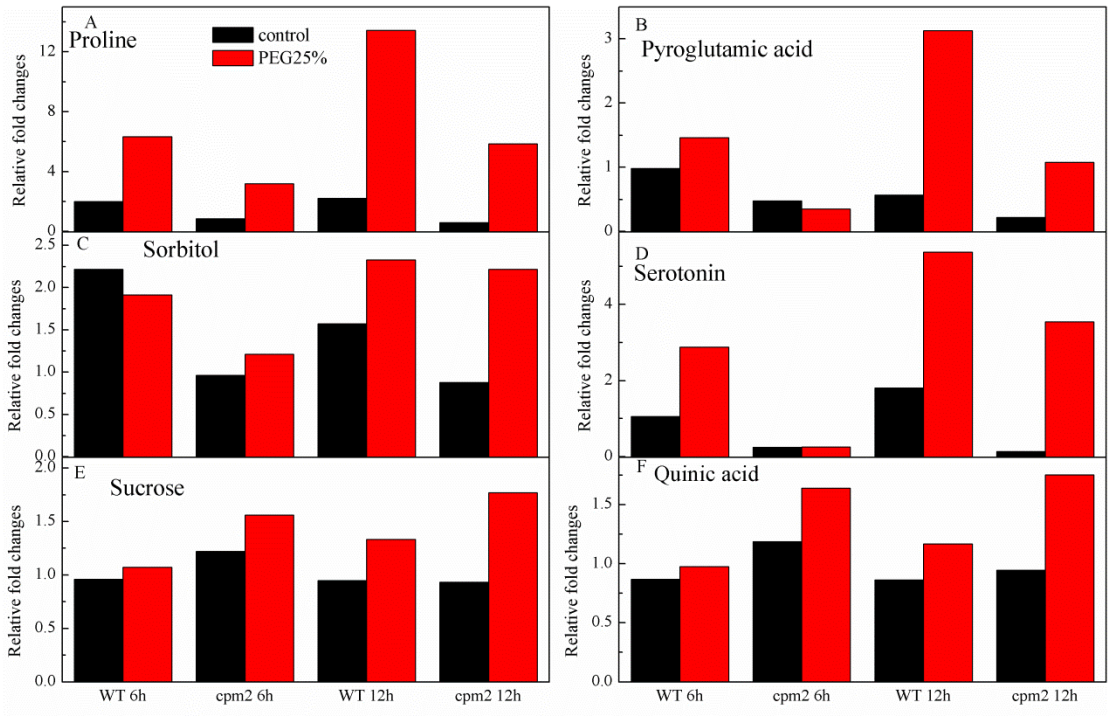

Figure 3.13. Proline, pyroglutamic acid, sorbitol, serotonin, sucrose, and quinic acid in shoots of wild type (WT) and JA biosynthesis mutants (*cpm2*) in response to osmotic stress (25% PEG 6000) after 6 h and 12 h. WT and *cpm2* were exposed to control (water, black bars) and osmotic stress (25% PEG 6000, red bars) treatment. The levels of (A) proline, (B) pyroglutamic acid, (C) serotonin, (D) serotonin, (E) sucrose, and (F) quinic acid were quantified relative to control plants (WT and *cpm2* at 0 time point, without any treatment). Values represent the mean of three independent experiments without error bar.

3.7 The function of JA is age-dependent

Previous researches of our laboratory suggested that *cpm2* showed stronger salt resistance than WT (Hazman et al., 2015; Kurotani et al., 2015). Furthermore, *cpm2* also had stronger drought resistance compared with WT (Dhakarey et al., 2017). Both results are contrary to those of the present study, especially the study of drought stress. However, Dhakarey's study investigates rice with at least 3-mo. maturity, which could already fruit. Our rice, on the other hand, was just 7- to 10-d-old. We studied whether JA had different effects in different growing periods in rice. To achieve this, we took a simple treatment. Specific experimental steps were as follows: first, rice was cultured

in a normal method for 7 d. Then, the 7-d-old rice was transformed into water (+ 0.344g/L MS) to culture for 1, 3, 5, and 7 d, respectively. After 1, 3, 5, and 7 d culture, rice with different ages were treated with 25% PEG, respectively. In the image, it can be seen that when rice cultured for 1, 3, and 7 d, WT had more tolerance to osmotic stress compared with *cpm2* (Fig. S7A, B, D), which indicated that JA contributed to defense against osmotic stress. However, when rice cultured for 5 d, *cpm2* showed more tolerance to osmotic stress (Fig. S7C), which indicated that JA may have a negative effect on defense to osmotic stress. This result proved our hypothesis that JA has different effects on rice in different age periods in rice. However, we are still unable to determine why the function of JA in defense against osmotic stress can change in such a short time (2 d). Further work on rice age and functions of JA in drought resistance may be requisite.

4. Discussion

The results demonstrated that JA can not only regulate plant growth and development, including germination, senescence, fruit ripening, growth of roots, development of pollen, formation of corm and tendril curling, but also participate in plant defense responses towards mechanical wounding, diseases, pests, temperature stress, salt stress, and other stress environments (Stintzi et al., 2001; Spoel et al., 2003; Schilmiller et al., 2007; Stein et al., 2008; Wang et al., 2008). It was found that when plants suffered from cold stress, the synthesis of endogenous JA is soon activated (Pedranzani et al., 2007). JA may accomplish the goal of improving plant resistance for cold by adjusting the transcription way of CBF (C-REPEAT BINDING FACTOR) and then upregulating cold-responsive genes downstream (Hu et al., 2013). In rice, it has been determined that the use of exogenous JA can improve the living condition of rice seedlings under salt stress, such as leaf water potential, photosynthetic rate, and maximum quantum yield of photosystem II (PSII). This function is also particularly obvious in salt-sensitive cultivar (Kang et al., 2005).

Although numerous studies identified the function of JA on cold and salt stress, very few reports seem to exist on the effects of JA in plant osmotic stress. Thus, in our present work, the objective was to elucidate the function of JA on defense against osmotic stress. Specifically, we intended to determine if JA contributes to resistance to osmotic stress and identify the potential mechanism.

4.1 Hormones are necessary in the process of resistance to osmotic stress

Plant hormones can greatly assist plants to improve adaptation to adversity stresses (Argueso et al., 2009; Santner and Estelle, 2009; Wang et al., 2009; Messing et al., 2010). Among these, one hormone that has been most studied is ABA. Of course, it is strongly associated with plant resistance. One of the regulation mechanisms is critical

in helping plants to better cope deal with osmotic stress. Specifically, the synthesis of ABA under osmotic stress is usually rapid, and will soon activate the expressions of ABA-related genes (Yamaguchi-Shinozaki and Shinozaki, 2006). This then leads to the closing of stomas, reduces plant water loss caused by transpiration, and finally achieves and maintains a better condition of moisture for plants (Wilkinson and Davies, 2010).

Seo et al. (2011) found that the transcription level of helix-loop-helix domain gene (OsbHLH148) would increase rapidly after treatment of MeJA and ABA. It would also increase under some abiotic stresses, such as drought, high salinity, low temperature, and wounding. Expressions of OsbHLH148 gene will give plants a strong ability for defending against drought stress. In this process, OsbHLH148, *OsJAZ*, and *OsCOI1* constitute a signalling module (Seo et al., 2011). Although there have recently been fewer related researches of JA and drought stress than those of ABA and drought stress, most reports still asserted that JA could improve plant resistance to drought stress (Alam et al., 2014).

4.1.1 JA mitigates osmotic stress symptoms

In the present work, we employed the AOC mutant *cpm2* and the corresponding WT plant Nihonmasari, and observed the response of these two genotypes to osmotic stress imposed by a chemical compound in a hydroponic system. We found that JA mitigates symptoms imposed by osmotic stress in rice, since *cpm2* seedlings were more strongly affected by the treatment. In addition, when both genotypes were subjected to strong osmotic stress, WT showed much more osmotic stress tolerance compared to *cpm2* (Fig. 3.1A-C). This phenomenon suggested that, on the one hand, young leaves were vulnerable to osmotic stress; on the other hand, the function of JA in defence against osmotic stress might be more relevant in young, developing tissues than in older ones where such difference became weaker (Fig. 3.1).

Since 25% PEG is a kind of very strong osmotic stress to both WT and *cpm2*, even WT shows stronger resistance than *cpm2*. However, either genotype of rice can hardly survive ultimately. For this, we studied the adaptations of two genotypes under weaker osmotic stress. We found that, under normal conditions (0%) or under 5% PEG treatment, *cpm2* grew bigger than WT (Fig. 3.2A). This result suggested that plants grow faster with the absence of JA. Considering the close connections between auxin and plant growth, we concluded that the fast growth of *cpm2* might be associated with auxin or GA. Previous experiments also found that some crosstalk existed between JA and auxin (Tiryaki and Staswick, 2002; Riemann et al., 2015). When rice was exposed to 5%-20% PEG, both WT and *cpm2* survived, or were at least healthy 3 d after treatment. However, the two genotypes seemed to become smaller under the stress condition. In other words, under low-concentration of osmotic stress, rice may decrease its growing speed to distribute resources into defending against osmotic stress to survive, in which JA might play a part. Moreover, similar to high-concentration PEG treatment, *cpm2* seemed to be more sensitive to osmotic stress than WT. WT also exhibited stronger resistance, since the decrease of WT was obviously less than that of *cpm2* (Fig. 3.2). The result of the present work indicated that JA mitigates osmotic stress symptoms irrespective of whether it was weak stress or lethal strong osmotic stress.

4.1.2 Differential increase of jasmonates in roots and shoots

When WT seedlings were treated with 25% PEG, jasmonates were strongly induced and accumulated very quickly in both shoots and roots (Fig. 3.3). Moreover, the jasmonate accumulation in roots preceded that in shoots. This increase in roots was transient; whereas, jasmonates accumulated in shoots over time. This indicates that there must be a basic difference of JA signalling in these two organs. After decreasing slightly at 3 and 6 h of stress treatment, JA and JA-Ile levels clearly rose at 24 h, indicating that the plant was suffering severely at this time point. Particularly in roots, the amount of OPDA increased approximately three-fold as compared to at 3 h of

stress. As an intermediate product of JA biosynthesis, OPDA can also be regarded as a signal, because OPDA itself can regulate gene expression (Stintzi et al., 2001; Taki et al., 2005). In roots, such late accumulation of jasmonates was not observed (Fig. 3.3B). We propose that, in roots, jasmonates are utilized in order to induce certain morphological and molecular changes towards adaption and more efficient water usage. Under persistent stress conditions, jasmonate levels did not change in the root, and thus are mainly used during a first reaction to this type of stress. In contrast, in the shoot, they probably assist the plant to adapt to the harsh environment, as they constantly increase, which correlates with the phenotype of larger tolerance to osmotic stress. During prolonged exposure to stress, the very high levels of jasmonates might also be indicative of damage.

Similarly to the hormonal changes, JA response gene expression was subsequently upregulated presumably to support defence and adaptation to osmotic stress. *OsJAZ4* and *OsJAZ11* genes were upregulated in WT shoots (Fig. 3.9A, B) at 1 h and 3 h of PEG treatment, respectively. In roots, *OsJAZ1* and *OsJAZ11* were found to be induced by osmotic stress at 1 h and 3 h, respectively (Fig. 3.9 A, B). Besides the JA response genes, several JA-biosynthesis genes were also induced in response to abiotic and biotic stress (Wasternack, 2014; Pratiwi et al., 2017). Our study revealed that most of the JA-biosynthesis genes that we examined were upregulated under osmotic stress. *OsAOS1*, *OsAOS2*, *OsAOC*, and *OsOPR7* were induced by osmotic stress in both WT shoots and roots (Fig. 3.8 A, B). Here, we noticed that these genes were upregulated longer in shoots than in roots, similarly to the pattern of hormonal content. At 3 h of the PEG treatment, these JA-biosynthesis genes were still upregulated in shoots, but not in roots, correlating well with hormonal content. Most of the JA-biosynthesis genes did not show any difference between the treatment and control in the *cpm2*, pointing to a JA-dependent transcriptional regulation of these genes under the conditions applied. However, it is noteworthy that *OsAOS1* was also upregulated obviously in *cpm2* after osmotic stress (Fig. 3.7D), which indicates that the transcript is partially regulated by a factor different from JA. *OsAOS2* was only upregulated 1 h

after the osmotic stress in WT shoots and roots, and not as strongly as *OsAOS1* (Fig. 3.7A-B). Surprisingly *OsJAR1* did not respond strongly to osmotic stress on the transcriptional level, indicating that, for this enzyme, posttranscriptional regulation might occur (Fig. 3.8C-D).

Taken together, regulation of hormonal content and expression of related genes correlated well and indicated that roots probably use jasmonate signals for immediate adaption, while shoots utilize the hormone for sustained signalling during continuous stress, culminating in hyper-accumulation as an indication for damage under prolonged severe stress conditions.

4.1.3 ABA and JA pathways respond to osmotic stress in parallel

ABA is the central signal which controls stomatal aperture and transcriptional activity in response to osmotic stress in plants (Fujita et al., 2011). In the present study, we also observed the time course of ABA in WT when exposed to osmotic stress. Similar to jasmonates, ABA increased and decreased earlier (1 h) in roots than in shoots when seedlings were subjected to osmotic stress (Fig. 3.4). Different from jasmonates, which levels were approximately equal in roots and shoots, levels of ABA were much lower in roots than in shoots (Fig. 3.3).

We found that levels of both jasmonates and ABA were induced earlier in roots than in shoots. This may be due to the fact that the roots were submerged in the PEG solution and therefore sensed the osmotic pressure first. In addition, the increased levels remained for a short time in roots; whereas, in shoots, levels increased later, but persisted for a longer time. Previous study suggested that root apices may act as a controlling unit, and vascular tissues may be responsible for the rapid information exchange between belowground (roots) and aboveground (shoots and leaves) organs (Baluška et al., 2004). This scenario requires rapid signalling between roots and shoots, and it was proposed that hormonal mediators could be involved (Urano et al.,

2014). The present study could support the viewpoint that roots may play such a role, as they remained comparatively unperturbed in terms of induction of stress hormones and switched them off quickly, even when exposed to a strong prolonged osmotic stress. Hence, the roots would remain less affected under stress and therefore fulfil an essential criterion for a controlling unit.

In both organs, the peak of ABA content was observed approximately 2 h later than that of JA/JA-Ile (Fig. 3.3). This suggested that the first hormonal signal to osmotic stress might be an increase in jasmonates, and raised the question of whether the accumulation of JAs in the early stage of osmotic stress might control a subsequent increase of ABA. Our study provides evidence that JAs might support plants to accumulate higher levels of ABA in shoots, as we found that *cpm2* mutants accumulated less ABA in shoots than the WT (Fig. 3.6 A). However, the content of ABA was higher in *cpm2* roots after 3 h of stress (Fig. 3.6 B). As LEA genes are generally induced in response to the accumulation of ABA (Lu et al., 2009; Fukao et al., 2011), we used *OsLEA3* as a marker gene for ABA. The expression pattern of this marker gene correlated well with the changes in hormonal contents in WT and *cpm2* (Fig. 3.10). Thus, both the hormone data and gene expression data demonstrated that JA interacted synergistically with ABA in rice shoots (Fig. 4.1). Previous studies also found that transient accumulation of JA was needed for ABA accumulation in *Arabidopsis* (Ollas et al., 2015) and citrus (Ollas et al., 2013) roots when exposed to drought stress. However, in rice, both hormonal pathways seem to be induced by osmotic stress more or less independently, as jasmonates were induced earlier than ABA, and ABA was induced even in the absence of JA. Moreover, in roots, ABA levels were higher in *cpm2*, indicating that JA represses ABA accumulation, although certainly not entirely. One of the possible mechanisms for this has been discussed above. Overall, the level of both hormones was much lower compared to the shoots, and the response was only transient. Hence, the roots, although directly exposed to osmotic stress, remain more or less unaffected on the level of accumulation of these stress hormones. This would enable them to act as a control unit of the plant which

can initiate effective countermeasures against the stress, as they are less harmed. In the future, it will be worthwhile to investigate whether such signalling occurs between roots and shoots during osmotic stress.

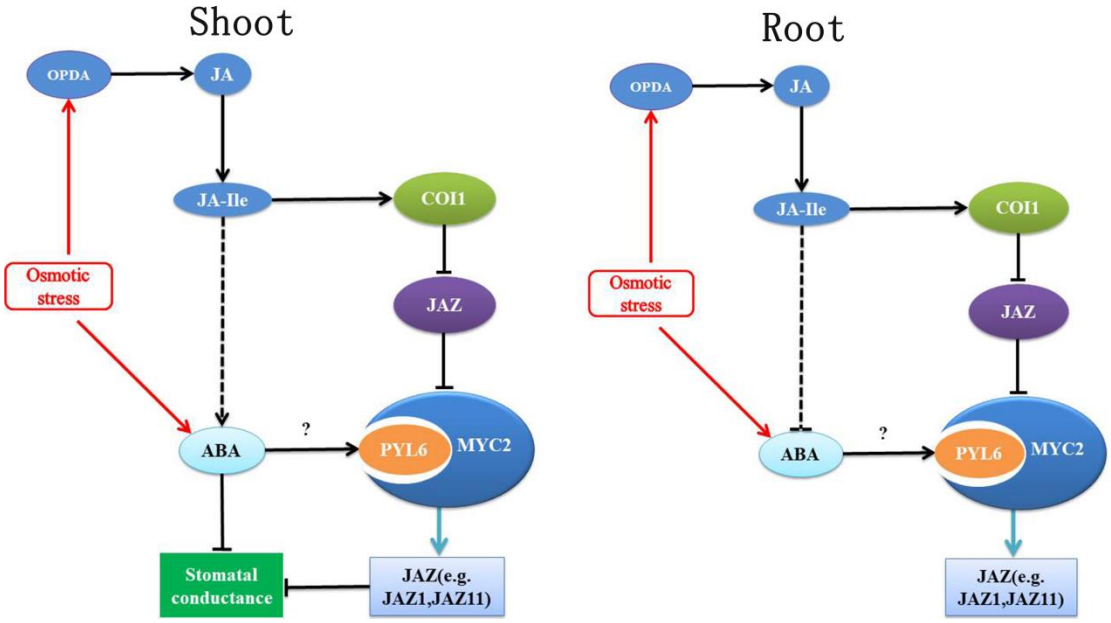

Figure 4.1. Proposed working model representing some possible mechanisms for how jasmonates contribute to osmotic stress adaptation. The observed phenotype of increased osmotic stress tolerance in wild type could be linked to the following possible mechanisms: when suffering from osmotic stress, rice roots received stress first, and then stress arrived in rice shoots. In rice roots, JA and ABA not only transport to shoots, respectively, but JA may also contribute to ABA transport from roots to shoots. In rice shoots, JAs were quickly synthesized, which induced upregulation of several JAZ (e.g., JAZ1, JAZ4, JAZ11) genes. Accumulation of JAs may further induce ABA synthesis at the later time point. The accumulation of ABA also induced PYL6 to bind to MYC2, which further upregulated the expression of several JAZ genes. Increased JA and ABA synthesis, which subsequently resulted in stomatal closure, may also help rice conserve more water in shoots and better adapt to osmotic stress.

4.2 Mechanism of JA-induced osmotic stress defense in rice

From previous studies, we first found that WT performed better in coping with PEG of different concentrations compared with *cpm2*. As a stress defense hormone, JA contributes to defense against osmotic stress in most cases, and it generally functions

as a positive regulator of stress tolerance. Then, by observing JA and ABA content in two genotypes (WT vs. *cpm2*) and expressions of related genes, the effects of JA in defending against osmotic stress in shoots and roots could be determined, as well as how JA and ABA work together in the process of defending against osmotic stress. Consequently, we aimed to elucidate about how JA assists plants to obtain a more effective mechanism of stress resistance. We investigated the third objective of our study: How does JAs contribute to defending against osmotic stress through morphological and physiological changes? In the following, we discuss several kinds of stress resistance mechanisms induced by JA.

Stomatal conductance

Leaf is the main organ of plant anabolism and transpiration, and stomas on leaf are an important pathway of plant gas exchange and water transpiration (Yao et al., 2006). Stomas originated from the activation of leaf epidermal cell (Nadeau and Sack, 2002). Then, cells nearby obtained the ability to divide, and most stomas were produced (Geisler et al., 2000; Serna and Fenoll, 2002). The size, quantity, and adjusting function of stomas have close connections with leaf photosynthesis, transpiration, and other physiological processes (Hetherington and Woodward, 2003; Casson and Gray, 2008).

ABA is a well-known plant hormone that can influence stomatal movement (Bright et al., 2006). Plants produce and accumulate excess ABA in guard cells under drought stress, which leads to the closing of stomas and thus retention of moisture (Thomashow, 2010; Hu et al., 2013; Miura and Tada, 2014). Usually, stresses can be transformed into ABA signals and further lead to activation of plant physiological and development processes (Soar et al., 2004). These physiological processes can help plants to better adapt to stress environments (Gibbs et al., 2010; Bailey-Serres et al., 2012). Of the different kinds of abiotic stresses, drought stress is the most common. Moreover, after suffering drought stress, ABA synthesis is one of the most rapid

responses of plants (Peleg and Blumwald, 2011). Plants then activate the expressions of ABA-related genes (Yamaguchi-Shinozaki and Shinozaki, 2006) and cause stomas to close, thus reducing water loss by decreasing transpiration (Wilkinson and Davies, 2010).

Considering that the degree of stomatal conductance is the main factor relating to the quantity of water loss, we found that stomas might constitute the key factors that caused WT and *cpm2* to adapt to water stress. Obviously, osmotic stress can rapidly reduce stomatal conductance of plant leaves, but the degree of reduction in WT is much greater than that in *cpm2* (Fig. 3.11). Since stomatal conductance of WT is much less than *cpm2*, it is easy to understand that WT can conserve more water compared with *cpm2* due to the low transpiration. Therefore, WT has stronger resistance against osmotic stress than *cpm2*. Because of the close relations between ABA content and stomatal conductance, we assert that the greater accumulation of ABA in WT might be a reason why WT can better adapt to osmotic stress.

Apart from ABA, JA is also found to greatly accumulate during drought stress, and plays a positive role in the processes of opening and closing stomas (Sarwat and Tuteja, 2017). Therefore, JA has been thought to have a very important function in drought stress response (Suhita et al., 2003; Suhita et al., 2004). In other words, besides low concentration of ABA content, a lack of sufficient JA might be another reason why *cpm2* cannot close stomas in time and thus cannot adapt to osmotic stress well. Other studies have shown cytoplasmic alkalinization in guard cells, activation of K-efflux and production of ROS (via AtRboHD/F) and NO (Evans, 2003), as well as slow anion channels (Suhita et al., 2003; Suhita et al., 2004; Munemasa et al., 2007) in conjugation with MeJA-mediated stomatal closure. Hossain et al. (2011) found reduced stomatal conductance in MeJA-induced Ca^{2+} oscillations in the guard cells of leaves where ABA biosynthesis is decreased (Hossain et al., 2011). Thus, MeJA and ABA are demonstrated to have a synergistic effect in inducing stomas opening and closing, and they cannot work alone, especially in the field of plant adaptation to

drought (Munemasa et al., 2007; Sarwat and Tuteja, 2017). So, in our study, we also assert that JA and ABA are likely to work together on stomas opening and closing in a synergistic manner.

ABA accumulates more in WT shoots than that in *cpm2*, and the biggest difference between the two genotypes is the ability to produce JA. Thus, ABA synthesis may be partially correlated with JA, which has been previously discussed. Since ABA could be produced greatly even in *cpm2*, JA might just be involved in one or some synthesis pathways, but not all pathways. Overall, the mechanism of JA adjusting stomatal conductance is somewhat complex. Specifically, on the one hand, JA might promote ABA synthesis and subsequently regulate stomatal conductance; on the other hand, JA or MeJA itself has the ability to regulate stomatal conductance. Of course, we recognize a greater synergistic effect than that of working alone. The decrease of stomatal conductance can assist plants to conserve more water in leaves and adapt well to osmotic stress.

ROS

Under low concentration, as a kind of second messenger signal molecule, ROS plays an essential part in controlling plant cell life activities. When plants suffer from osmotic stress, ROS content rises and is delivered to activate different signal pathways, promoting plant issues to produce a series of defense responses (Neil et al., 2002; Vranova et al., 2002). Under high concentration, however, ROS is extremely deleterious to plants. When the ROS level is beyond that which the defense mechanism can handle, cells will be under oxidative stress, which results in lipid peroxidation, protein oxidation, nuclear acid damage, enzyme inactivation, and activation of the death of programmed cells (Apel and Hirt, 2004; Møller et al., 2007). In plants, the elimination of ROS is made of enzymatic and non-enzymatic antioxidant systems together (Ali et al., 2006).

Previous studies suggested that exogenous application of JA and MeJA could protect plants from damage induced by drought stress by improving plant oxidation resistance (Bandurska et al., 2003). When plants suffer drought stress, the metabolism of antioxidant ascorbate would be influenced by JA, as well (Ai et al., 2008). Other studies also indicated that exogenous JA could improve plant resistance for drought stress, since JA increased the activity of antioxidant enzymes, thus decreasing plant oxidative damage (Nafie et al., 2011).

In our study, we found that O_2^- and MDA content were higher in *cpm2* than that in WT. In other words, *cpm2* would accumulate more ROS than WT (Fig. 3.12) when facing osmotic stress. We also determined that O_2^- or MDA content was very high, so it was likely that ROS was a kind of poisonous substance instead of a signal molecule. Consequently, we assert that JA might adapt to osmotic stress by regulating the accumulation of ROS content under osmotic stress. The reasons why JA could decrease ROS content in response to osmotic stress were various, but mainly comprise two mechanisms: less come and more go. Specifically, JA may inhibit the synthesis of ROS or JA may induce the expression of anti-oxidative enzymes. Previous studies indicated that JA increased the activity of antioxidant enzymes, thus decreasing plant oxidative damage and improving plant resistance for drought stress (Nafie et al., 2011). In our study, we supposed that JA might realize the goal of eliminating oxygen radical and reducing plant oxidative damage by activating antioxidant enzymes. However, as we do not possess further data of anti-oxidative enzymes (e.g., SOD, catalase, or peroxidase), the mechanism of JA-mediate decline of ROS production remains undetermined. Further study could focus on this specific issue.

Trade-offs and amino acid synthesis

Jasmonates may also influence trade-offs (Gould et al., 2008), which refers to the increase of one trait that coincides with a decrease of another (Machado et al., 2017).

JAs participate in most of the biosynthetic pathways of secondary metabolites (Wasternack and Strnad, 2017). Amino acids are not only the necessary substrate of protein synthesis, but also play an important role in plant primary and secondary metabolism (Oren and Gunde-Cimerman, 2007). Some amino acids are precursors of secondary metabolite (e.g., hormones, plant defense-related substances, etc.) synthesis (Croteau et al., 2000). Amino acids improve plant adaptive responses mainly by participating in changing some physiological metabolism in plants or adjusting the expression of related genes and key enzymes activities (Szabados and Savoure, 2010). At present, studies on amino acids' participation in plant stress adaptation mainly include reducing heavy metal toxicity, improving drought, salt stress resistance, regulating osmotic potential and cationic transport, and eliminating reactive oxygen injury (Rai, 2002; Sharma and Dietz, 2006; Szabados and Savoure, 2010; El-Samad et al., 2011). From the discussions above, there are many kinds of amino acids, and the functions of amino acids in plants are varied.

In our study, many amino acids also accumulate more in WT than that in *cpm2*. Since most amino acids accumulate more in WT, we also believe that the synthesis or decomposition of these amino acids is related to JA. Considering that JA is involved in the biosynthesis of almost all secondary metabolites (Wasternack and Strnad, 2017), we also tend to conclude that JA is involved in the synthesis of many amino acids, which play a role in plant resistance to osmotic stress. Of all amino acids, proline is regarded as an osmotic regulator under stress (Maggio et al., 2000), and it can also act as protection against oxidative stress (Sorkheh et al., 2012; Zhang et al., 2014). Thus, the accumulation of proline may help plants adjust osmotic pressure and inhibit oxidative stress, which subsequently enables better adaption to osmotic stress. Our study also demonstrated that WT can accumulate more proline and can be rapidly induced by osmotic stress. The accumulated proline can reduce the water potential of plants and maintain low osmotic pressure, so that plants can absorb more water from the surrounding environment instead of losing water. Expressions of *OsP5CS* in two genotypes exhibit good consistency with the results of proline when rice was exposed

to osmotic stress.

Phenylalanine ammonia-lyase (PAL) related gene expressions are closely connected with phenylalanine. The results showed that WT accumulated phenylalanine more easily compared with *cpm2*, while phenylalanine was very sensitive to osmotic stress and could rapidly accumulate under stress. In order to study the role of phenylalanine in plant osmotic stress, we also investigated the related PAL gene. PAL is a conserved homotetrameric protein which is a key enzyme in the phenylpropanoid pathway in higher plants (Bowles, 1990; Bate et al., 1994; Reichert et al., 2009; Rawal et al., 2013). Products of this biosynthesis pathway include soluble phenolics, flavonoids, and lignin, which may contribute to stress defense (La Camera et al., 2004; Vogt, 2010; Tonnessen et al., 2015). Since lignin is an essential component of plant cell walls, the amount of lignin can affect the strength of cell walls. In our study, we also found that *cpm2* grows more rapidly than WT under normal conditions, but it does not seem as robust as WT (Fig. 3.1). Therefore, the reduction of PLA and lignin caused by JA deficiency may a reason why *cpm2* appears "fragile". L-tryptophan decarboxylase (TDC) has been investigated widely because it plays a critical role in the biosynthesis of secondary metabolites in plants (Kang et al., 2007). Besides *OsP5CS* and *OsPAL4*, *OsTDC1* and *OsTDC2* were also found to be higher in WT than in *cpm2*. Therefore, compared with *cpm2*, WT is more likely to synthesize secondary metabolites. It is known that most secondary metabolites have positive effects on plant resistance to stress. Therefore, more secondary metabolites in WT may also be one of the key factors leading to its superior adaptation to osmotic stress.

The measurement of amino acid content showed that most amino acids were easier to synthesize in WT, and it seems that most of these amino acids are active in plant defense. So, the rapid growth of *cpm2* might be at the expense of its resistance to stress. Therefore, we assert that one of the functions of JA is to balance the allocation of plant resources. JA can promote synthesis of a large number of amino acids and secondary metabolites, while these substances, in turn, greatly increase plant

resistance to stress, rather than allowing plants to grow indefinitely.

Morphology adaptation

From the above discussions, we have shown that JA can help plants to reduce stomatal conductance in order to conserve more water in the short-term under osmotic stress. However, when plants are exposed to persistent osmotic stress, they need to make some more physiological and morphological adjustments in order to adapt. Previously, we have stated that plants may be able to adapt to stress through synthetic amino acids and secondary metabolites. Here, we discuss changes in morphology. The root system is the main organ that absorbs water (Patten and Glick, 2002). Under osmotic stress, in addition to reducing water loss through decreasing stomatal conductance, plants also need to increase their ability to absorb water to maintain normal growth. In drought environments, because plants need to absorb water only through the absorption of groundwater, most desert plants are more apt to grow deeper than to put root construction on the surface of the soil (Benjamin and Nielsen, 2006). Our study found that, under weak osmotic stress, the increasing speed of crown roots of WT was faster than that of *cpm2* (Fig. 3.2F). In other words, JA is more able to help plants grow more roots rather than make the roots grow longer. In our study, this is because rice only needs to grow more roots to absorb more water, and longer roots would not benefit survival. Therefore, we think that JA may assist plants to adapt to long-term osmotic stress by adjusting the morphological features of plants, especially those of roots.

4.3 Age-dependent JA induced defense against osmotic stress in rice

The results of the current study clearly demonstrate that JA can assist plants to better adapt to osmotic stress, and many possible mechanisms have been discussed. In fact, however, the relationship of JA to osmotic stress may not be as simple as we assumed. In our study, it was also observed that JA had different effects on the resistance to

osmotic stress of rice when the growth time of rice differed. We found that when WT and *cpm2* were cultured in water for 5 d, *cpm2* possessed stronger resistance to osmotic stress than WT (Fig. S7). In other words, when the rice grows to a certain stage, JA may play a negative role in the defense of osmotic stress. We were also surprised by this result, and are not certain concerning the reasons why the effects of JA on rice resistance to osmotic stress were so different in only 2 d. It is also speculated that the plant faces different tasks at different stages of growth. We divide the growth of plants into three parts: vegetative growth, reproductive growth, and defense. When rice grows to about 3 mo., plants need to devote most of their resources to reproductive growth, and *cpm2*, which lacks JA, cannot form seeds. Therefore, *cpm2* at this stage does not need to allocate resources to reproductive growth, but has a stronger ability to resist drought stress, which was also found in our previous study (Dhakarey et al. 2017). In the early stage of rice growth, reproductive growth is not the main factor of resource consumption, because JA helps plants to invest more resources into defense. Thus, JA plays a positive role in defense against osmotic stress in young rice plants. Therefore, we suspect that JA may be a key to maintain the balance of resource allocation. Since the allocation of resources may differ at each stage of plant growth, the adjustment function of JA to osmotic stress may also be varied over time.

In summary, our study clearly shows that JA can help plants better adapt to various intensities of osmotic stress. Moreover, this function varied among different stages of growth, and sometimes even the opposite result will occur. In the early stages of plant growth, when reproductive growth does not need to be considered, vegetative growth and defense occupy the most important aspects of plant life. At this time, JA inhibits the rapid growth of plants, thereby investing the remaining resources into the plant's defense system to achieve better adaptation to osmotic stress. This ability of JA might be achieved through interaction (crosstalk) with other growth hormones, such as auxin, cytokinin, and GA. We also conclude that JA can promote the synthesis of many amino acids and secondary metabolites that are related to osmotic stress, thus

assisting the plants to have a greater ability to defend against osmotic stress. When plants are subjected to osmotic stress, JA and ABA can accumulate in a short time as a hormone, and induce the expression of related genes, produce proteins that are related to osmotic stress resistance, eliminate adverse conditions, and ultimately adapt well to osmotic stress. Although ABA can be synthesized separately under stress conditions in the absence of JA, the presence of JA can significantly promote the accumulation of ABA in rice shoots, which indicates that JA may be involved in one or part of the biosynthetic pathway of ABA, but not all of the pathways. Since we found that JAs' increase in roots was transient, whereas JAs accumulated in shoots over time, we propose that, in roots, jasmonates are utilized in order to induce molecular signals to arouse the defense system in plants. In contrast, in the shoot, jasmonates probably help the plant to adapt to harsh environments through certain morphological and physiological changes towards adaption and more efficient water usage. Both ABA and JA synthesis under osmotic stress can promote the reduction of stomatal conductance in rice. It is likely that JA and ABA work together on stomas opening and closing in a synergistic manner. In addition, the accumulation of JA and ROS is related; specifically, the increase of JA may correlate with the increase of ROS content, which is at a high level and most likely a toxic substance. It is also not clear whether the decrease in ROS is caused by the blocked synthesis that is related to JA, or the mechanism of elimination of ROS is augmented in the presence of JA. Finally, from a morphological perspective, JA promotes an increase in the amount of root system, which resulted in the increase of root surface area, which subsequently may lead to the increase of water absorption ability and more resistance to osmotic stress. Finally, JA-mediated defense against osmotic stress in plants is quite complicated. What makes the situation even more complex is that JA-mediated adaptation to osmotic in rice varies among different stages.

5. Outlook

As our study demonstrated, JA has different osmotic stress resistance to rice at different growth stages. However, we only described this phenomenon in our study. In following studies, we feel that it is necessary to further study the relationship between age and JA. We are particularly interested in the conditions under which JA has a negative effect on rice resistance (when and why). Moreover, changes in the content of many amino acids under osmotic stress were measured in our study. Most of these amino acids accumulated more in WT and some more in *cpm2*. Apart from the fact that the functions of amino acids, such as proline, have been studied thoroughly, it is not yet clear what role a large proportion of amino acids plays in the process of osmotic stress. So, much work can be done to determine the role of these amino acids in osmotic stress. In addition, one of the synthetic pathways of ABA in our study may be related to JA, but there is no indication of JA's involvement in ABA synthesis in the current study. Further research could explore the role of JA in ABA synthesis and how JA affects ABA synthesis.

6. Appendix

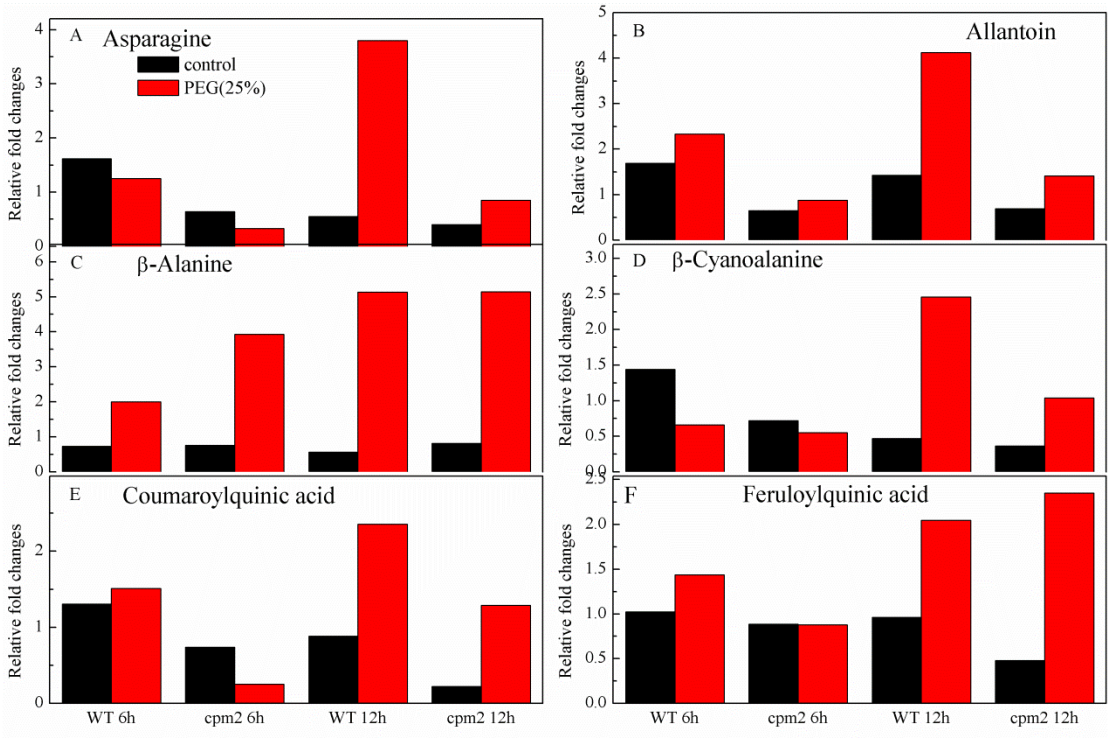

Figure S1. Asparagine, allantoin, β-Alanine, β-Cyanoalanine, coumaroylquinic acid, and feruloylquinic acid in shoots of wild type (WT) and JA biosynthesis mutants (*cpm2*) in response to osmotic stress (25% PEG 6000) after 6 h and 12 h. WT and cpm2 were exposed to control (water, black bars) and osmotic stress (25% PEG 6000, red bars) treatment. The levels of (A) asparagine, (B) allantoin, (C) β-Alanine, (D) β-Cyanoalanine, (E) coumaroylquinic acid, and (F) feruloylquinic acid were quantified relative to control plants (WT and cpm2 at 0 time point, without any treatment). Values represent the mean of three independent experiments without error bar.

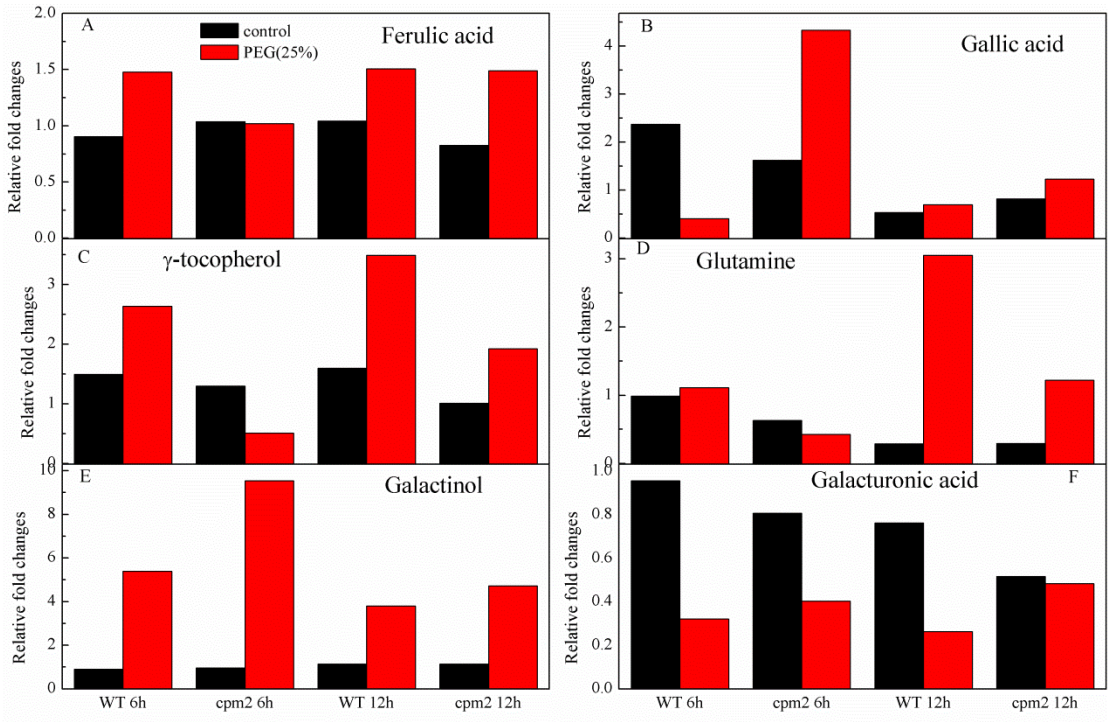

Figure S2. Ferulic acid, gallic acid, γ-tocopherol, glutamine, galactinol, and galacturonic acid in shoots of wild type (WT) and JA biosynthesis mutants (cpm2) in response to osmotic stress (25% PEG 6000) after 6 h and 12 h. WT and cpm2 were exposed to control (water, black bars) and osmotic stress (25% PEG 6000, red bars) treatment. The levels of (A) ferulic acid, (B) gallic acid, (C) γ-tocopherol, (D) glutamine, (E) galactinol, and (F) galacturonic acid were quantified relative to control plants (WT and cpm2 at 0 time point, without any treatment). Values represent the mean of three independent experiments without error bar.

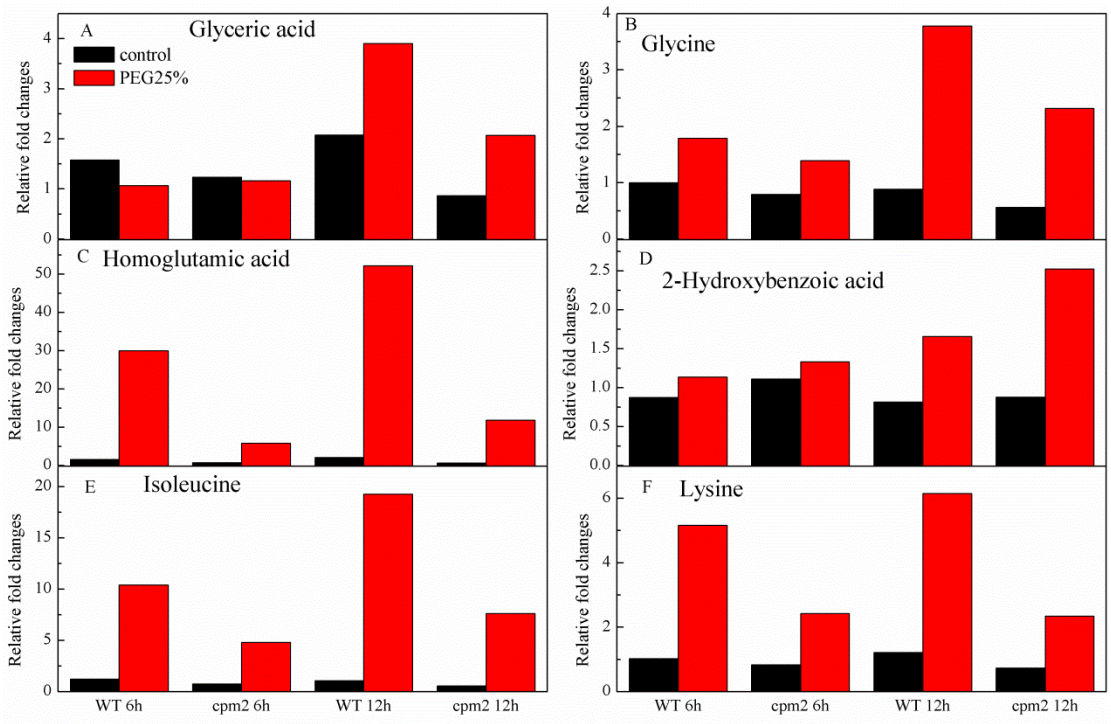

Figure S3. Glyceric acid, glycine, homoglutamic acid, 2-hydroxybenzoic acid, isoleucine, and lysine in shoots of wild type (WT) and JA biosynthesis mutants (cpm2) in response to osmotic stress (25% PEG 6000) after 6 h and 12 h. WT and cpm2 were exposed to control (water, black bars) and osmotic stress (25% PEG 6000, red bars) treatment. The levels of (A) glyceric acid, (B) glycine, (C) homoglutamic acid, (D) 2-Hydroxybenzoic acid, (E) isoleucine, and (F) lysine were quantified relative to control plants (WT and cpm2 at 0 time point, without any treatment). Values represent the mean of three independent experiments without error bar.

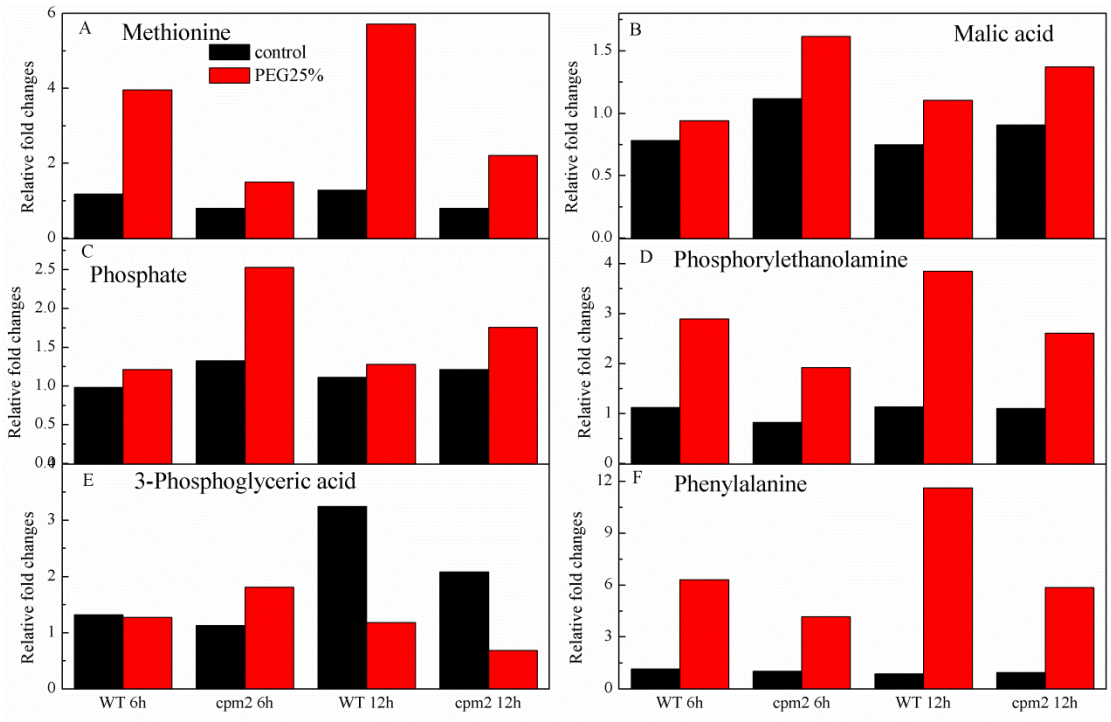

Figure S4. Methionine, malic acid, phosphate, phosphorylethanolamine, 3-phosphoglyceric acid, and phenylalanine in shoots of wild type (WT) and JA biosynthesis mutants (cpm2) in response to osmotic stress (25% PEG 6000) after 6 h and 12 h. WT and cpm2 were exposed to control (water, black bars) and osmotic stress (25% PEG 6000, red bars) treatment. The levels of (A) methionine, (B) malic acid, (C) phosphate, (D) phosphorylethanolamine, (E) 3-phosphoglyceric acid, and (F) phenylalanine were quantified relative to control plants (WT and cpm2 at 0 time point, without any treatment). Values represent the mean of three independent experiments without error bar.

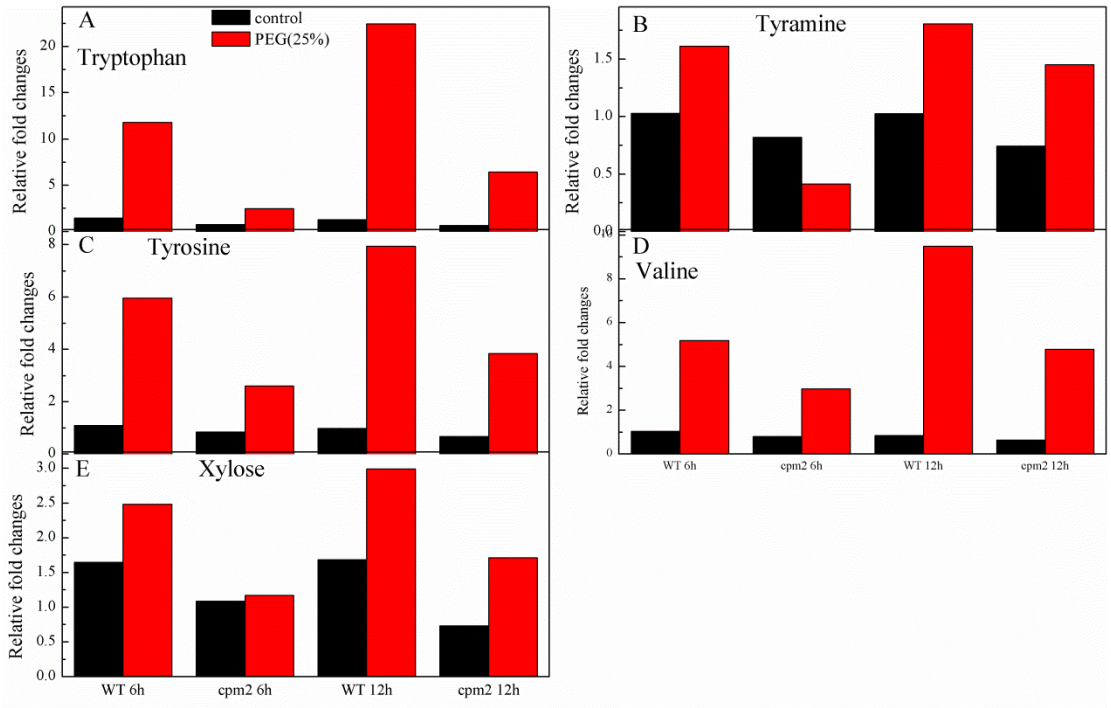

Figure S5. Tryptophan, tyramine, tyrosine, valine, and xylose in shoots of wild type (WT) and JA biosynthesis mutants (cpm2) in response to osmotic stress (25% PEG 6000) after 6 h and 12 h. WT and cpm2 were exposed to control (water, black bars) and osmotic stress (25% PEG 6000, red bars) treatment. The levels of (A) tryptophan, (B) tyramine, (C) tyrosine, (D) valine, and (E) xylose were quantified relative to control plants (WT and cpm2 at 0 time point, without any treatment). Values represent the mean of three independent experiments without error bar.

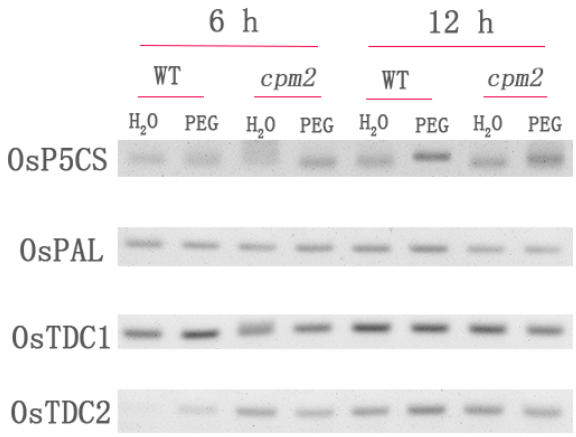

Figure S6. Transcriptional regulation of selected secondary metabolites related genes in response to osmotic stress (25% PEG 6000) in shoots and roots of wild type (WT) and JA biosynthesis mutants (*cpm2*). WT and *cpm2* have been exposed to control (water, black bars) and osmosis stress (25% PEG 6000) for 6 h and 12 h.

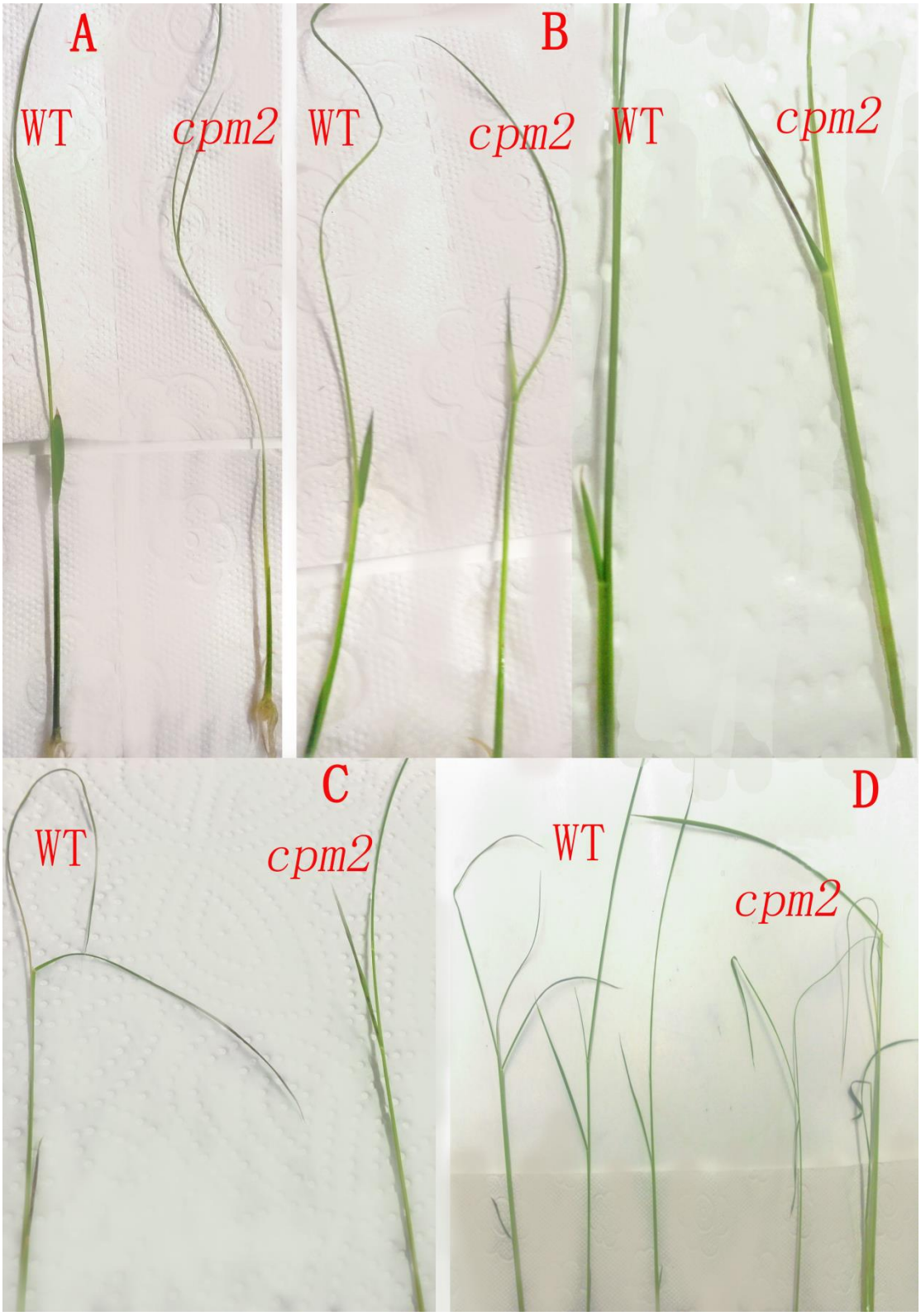

Figure S7. Different ages of rice (WT and *cpm2*) in response to osmotic stress. The rice was first incubated in 0.4% phytoagar medium (+ 0.344 g/L MS) for 7 d. Then, rice was transferred to water (+ 0.344 g/L MS) for (A) 1 d, (B), 3 d, (C) 5 d, (7) 7 d. Later, rice plants were treated with

25% of PEG6000 for 24 h. In our study, when rice was cultured in water (+ 0.344 g/L MS) for 5 d, *cpm2* showed more tolerance to osmotic stress; whereas; in other time points, WT showed more tolerance to osmotic stress. The experiment was repeated four times.

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