# Adaptation to drought in rice -

# Dissecting the role of Jasmonates for the response to drought in rice using a mutant approach

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Karlsruhe, im June 2016

Rohit Dhakarey

‘Look at the sky. We are not alone. The whole universe is friendly to us and conspires only to give the best to those who dream and work.’

- **Dr. APJ Abdul Kalam**

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# Contents

# ABBREVIATIONS

***cpm2*:** coleoptile photomorphogenesis 2

**OA:** Osmotic Adjustment

**WUE:** Water Use Efficiency

**ABA:** Abscisic Acid

**ABC:** Ammonium bicarbonate

**SMC:** Soil Moisture Content

**JA:** Jasmonic Acid

**LOX**: Lipoxygenases

**AOC:** Allene Oxide Cyclase

**OPDA:** 12-Oxo-Phytodienoic Acid

**OPR7:** 12-Oxophytodienoate Reductase

**ILE:** Isoleucine

**JAR1:** Jasmonate Resistant 1

**COI1:** Coronatine Insensitive 1

**TCA:** Trichloroacetic Acid

**2-DE:** Two-Dimensional Electrophoresis

**LC-MS/MS:** Liquid Chromatography Combined With Tandem Mass Spectrometry

**MS:** Mass Spectrometry

**TMT:** Tandem Mass Tag

**WW:** Well-Watered

**DS:** Severe Drought Stressed

**ROS:** Reactive Oxygen Species

**DEP:** Differentially Expressed Proteins

**Ψw:** Water potential

# Zusammenfassung

Phytohormone sind chemische Botenstoffe in Pflanzen, die unterschiedliche Reaktionen von Pflanzen, zum Beispiel auf Trockenstress, stark beeinflussen. Phytohormone unterstützen Pflanzen bei der Regulation von Wachstum und Entwicklung auch unter solchen ungünstigen Bedingungen. Eines dieser wichtigen Phytohormone ist die Jasmonsäure (JA). Über ihre Funktion für die Reaktion auf Trockenstress ist wenig bekannt, wie auch über die Vernetzung mit anderen Phytohormonen wie zum Beispiel Abscisinsäure (ABA), welche eine außerordentlich wichtige Funktion für Trockenstressadaptation hat. Im Vorfeld wurde bereits gezeigt, dass die Reismutante *cpm2*, die defekt in der Funktion eines Schlüsselenzyms der JA-Biosynthese, der Allenoxidcyclase (AOC), ist, weniger empfindlich für Salzstress ist. In der vorliegenden Studie wurden Veränderungen von physiologischen Eigenschaften und im Wurzelproteom von *cpm2* und Wildtyp (WT) Pflanzen analysiert. Die stomatäre Leitfähigkeit von *cpm2* war unter Trockenstress geringer als im WT, was mit einer erhöhten Konzentration von ABA im Spross korrelierte. Unter Trockenstress wies *cpm2* eine höhere Wassernutzungseffizienz (engl. water use efficiency, WUE) als der WT auf, was auch auf eine verbesserte Trockenheitstoleranz hinweist. Das Wurzelsystem von *cpm2* war sowohl unter Kontroll- als auch mittelstarken Stressbedingungen besser entwickelt als im WT. Um festzustellen, ob Wurzeln von *cpm2* und WT auf molekularer Ebene unterschiedlich auf Trockenstress reagieren, wurde das Wurzelproteom in einem Tandem Mass Tag (TMT) Ansatz untersucht. Auf Proteinebene wurde AOC ausschließlich im WT gefunden und war dort bei Trockenstress reichlich vorhanden, während AOC in *cpm2* nicht detektiert wurde. Ein anderes Protein, OPDA Reductase 7 (OPR7), das auf AOC folgende Enzym im JA-Biosyntheseweg, akkumulierte in *cpm2* unter Trockenstress, während die Menge im WT abnahm. Diese Ergebnisse weisen darauf hin, dass OPDA, eine Vorstufe von JA, und nicht JA selber unter Trockenstress akkumuliert. Die Analyse weiterer differentiell exprimierter Proteine zeigte, dass *cpm2* unter Trockenstress einen aktiveren ROS-Stoffwechsel und eine bessere ROS-Entgiftung aufweist. Außerdem waren in Wurzeln von *cpm2* Proteine häufig, die im funktionellen Zusammenhang mit Zellwandumbau und Zellwachstum stehen. Diese Ergebnisse zeigen, dass JA ein negativer Regulator der Trockenheitstoleranz ist, da sie für die Stressadaptation wichtige morpho-physiologische und molekulare Änderungen hemmt und Hemmung der JA-Biosynthese zu einer verbesserten Trockenheitstoleranz von Reis führen.

# Abstract

The various adaptive responses of drought stressed plants are strongly influenced by chemical messengers called phytohormones, which help the plants to regulate growth and development also in adverse conditions such as drought. One such important phytohormone is jasmonic acid (JA). However, little is known about its direct involvement in drought response or about its cross-talk to other phytohormones such as abscisic acid (ABA), which is intricately involved in drought stress adaptations. Previously, it was shown that the rice mutant *cpm2*, impaired in the function of allene oxide cyclase (AOC), a key enzyme in JA biosynthesis, was less sensitive to salt stress. In the present study, comparative changes under drought in some physiological traits and in the root proteome of *cpm2* and the wild type (WT) were analyzed. When the stomatal conductance in WT and *cpm2* under drought condition was measured, *cpm2* had lower stomatal conductance under drought stress as compared to WT and this also correlated with increased ABA levels in shoots. Under drought, higher Water Use Efficiency (WUE) in *cpm2* as compared to WT also indicated improved drought tolerance. Importantly, roots of *cpm2* were better developed under both control and moderate drought stress. To assess if the roots of *cpm2* and WT respond differentially to drought at the molecular level, root proteome analysis was undertaken using Tandem Mass Tag (TMT) approach. At the protein level, AOC was unique to WT and highly abundant under drought. This confirmed the lack of AOC in *cpm2*. Another protein, OPDA reductase (OPR7) which is downstream of AOC in JA biosynthesis pathway became more abundant in *cpm2* while its amount decreased in the WT in drought stress. These results suggest that OPDA, a precursor of JA, and not JA itself is accumulating in response to drought stress. Analysis of other differentially expressed proteins revealed more active ROS detoxification and metabolism in *cpm2* under drought. In addition, a number of proteins involved in pathways related to cell wall remodeling and cell growth were also abundant in *cpm2* roots. These results suggested that JA signaling might negatively influence drought tolerance by orchestrating a block on critical morpho-physiological and molecular changes necessary for stress adaptation. Mutant analysis suggested benefits of blocking JA synthesis which might be useful for improving drought tolerance in rice.

# 1. Introduction

Plants are vital for the balance of nature and in humans' wellbeing. Green plants, i.e., those synthesizing chlorophyll can manufacture their own food and give off oxygen in the phenomenon called photosynthesis during which water and carbon dioxide react chemically by the energy of light. For nearly all animals which cannot manufacture their own food, plants are the main source of food and metabolic energy. Rice (*Oryza sativa*) is the most important plant for human food security, as it is the staple food to more than half of the world's population. In addition to that, it is a model crop species because of the knowledge of its genome and its synteny with other crops (Cotsaftis and Guiderdoni, 2005). Plants are sensitive organisms that cannot run away from unfavorable conditions. Because of this reason, it becomes imperative to look at the immense variety of responses with which plants can react to a continuously changing environment. Stress is usually defined as any form of external factor that exerts disadvantageous effect on the plant. Mostly, stress is measured in relation to overall plant survival, reduction in yield or growth (biomass accumulation), or the primary assimilation process ($CO_2$ and minerals uptake), which are related to overall growth (Taiz and Zeiger, 2002). Drought is probably the most severe constraint for the productivity and quality of a crop among all environmental factors combined, compromising economic output and human food supply (Roche *et al.,* 2009).

## 1.1 Definition of Drought

Drought can be expressed as a period of below-normal precipitation that limits plant/crop productivity by imposing a water deficit, and thus bringing a reduced water potential in the plant (Verslues *et al.,* 2006). It is estimated that about 28% of the world's soil is constantly affected by drought, and up to 50% is affected repeatedly because of the poor water holding capacity shallowness of fields, and other factors (Salekdeh *et al.,* 2009).

Drought is a normal, recurring feature of climate, although it is incorrectly considered as a rare and random event. It is different from aridity, which is rather limited to regions of low rainfall regions and is a permanent component of climate. Drought should be contemplated relative to some long-term average conditions of equilibrium between precipitation and evapo-transpiration (i.e., evaporation + transpiration) in a specified area. It is also linked to the timing (season of occurrence, delays in the start of the rains, occurrence of rains is

related to principal crop growth stages) and the efficacy (i.e., intensity of rainfall, number of rainfall events) of the rains (Wilhite and Glantz, 1985).

Drought is a redolent term. It comes with implications of severe financial hardship among farmers in rich countries, to malnutrition and famine, among farmers in third world countries. If it prolongs, it can lead to major social turbulence, mass migration and desertification, not only leading to sense that the affected region is desolated by its former inhabitants, but also because over-used farm land may become so resourceless that it can no longer support human occupation even after the prolonged drought is over (Mueller *et al.,* 2005).

### 1.2 Drought endurance mechanisms in plants

Mechanisms that plants utilize to fight with drought can, more or less innately, be classified into 1) drought avoidance and 2) drought tolerance mechanisms. A complex combination of these defines drought resistance in its physiological context according to Levitt (1972). Each of these coping strategies is described in this section :

#### 1.2.1. Mechanisms related to drought avoidance

Drought-avoiding plants have the capability to complete their entire life cycle without being getting severe water deficient. Some ephemeral plants have a shortened life cycle that can be completed during a short span of rainy season. Other plants exhibit adaptations to increase water uptake and reduce water loss and therefore avoiding the exhausting consequences of drought that other plants might feel (Verslues *et al.,* 2006). Accordingly, evolving a more extensive root system is a drought-avoiding strategy. It is an almost universally accepted observation that the root:shoot ratio increases with drought stress. Greater increase in root weight may be due to a greater density or depth of roots (Turner, 1979). Under drought stress, new root development extends more deeply into moist soil zones. As drought stress progresses, the upper soil layers usually dry first. Thus shallower roots are quite common in wet soils as compared to deeper roots systems in dry soils (Taiz and Zeiger, 1998). The possession of a deeper and thicker root system, which allows better access to water deep seated in the soil is considered important in determining drought resistance in rice (Price *et al.,* 2002) and *Arabidopsis thaliana* (Xiong *et al.,* 2006). Therefore,

greater root growth and improved morphological development can result in drought avoidance. *DEEPER ROOTING 1* (*DRO1*) is a good example in this context. (Uga *et al.,*2013). It is a rice quantitative trait locus controlling root growth angle. *DRO1* is negatively regulated by auxin and it is involved in cell elongation in the root tip region that causes asymmetric root growth and downward curving of the root in response to gravity. Higher expression of *DRO1* leads to an increase in the root growth angle, whereas roots grow in a more downward direction. It is also shown by introducing *DRO1* into shallow-rooting rice cultivar by backcrossing enables the resulting line to avoid drought by increasing deep rooting. The resulting new line maintains high yield performance under drought conditions relative to the recipient cultivar.

Evolvements in shoots also play a vital role in drought stress responses. At the start of the dry season, a desert plant *Zygophyllum qatarense* responds to drought stress with a leaf polymorphism in which it replaces the wet-season foliage with unifoliate, xeromorphic leaves. As the dry season continues, the plant ceases the extension in its leaf area substantially (Sayed, 1996).

During drought stress, leaves of many plants frequently wilt (or roll, in the case of rice), and this response massively reduces the perception of radiation, thereby hampering the potential increase in leaf temperature arising from stomatal closure and inhibiting further development of leaf water deficit (Turner, 1979). Severe drought stress may lead to increased levels of abscisic acid (ABA) and subsequent leaf abscission, thereby limiting transpirational demand. Such developmental changes within a plant during drought stress are important morphological drought-avoiding adaptations that help the plant to maintain water potential at some functional level in the midst of potential water limitation (Blum 2005).

### 1.2.2 Mechanisms related to drought tolerance

As drought stress progresses and becomes more intense, it becomes increasingly more difficult for the plant to avoid dehydration; and mechanisms that allow plants to withstand reduced water content become massively important. Drought tolerance can be defined as the ability of plants to continue to functionally active despite at lowered tissue water potentials. Drought-tolerating mechanisms often involve the maintenance of turgor by

accumulation of solutes and/or desiccation tolerance by protoplasmic resistance (Jones *et al*., 1981). “Desiccation tolerant” plants can recover from a fully air-dried state (Vicre et al., 2004) but when dehydrated, these plants are in a metabolically dormant, or cryptobiotic, state. Mesophytic plants, including all crop plants, lack the propensity to enter the cryptobiotic state. Moreover, mesophytes typically cannot recover from severe (approximately 50% or greater) decreases in their water content (Verslues *et al.,* 2006). However, many plants such as resurrection plants have potential to tolerate significant water loss, while maintaining metabolic activity (Bartels *et al.,* 2006).

### 1.2.3 Morpho- physiological changes associated with drought resistance

Plants face drought stress either when the water supply to roots becomes limited or when the transpiration rate becomes higher than the rate of uptake of water. It severely affects growth, development and ultimately the yield of rice plant. When drought stress progress, plants react by decreasing or ceasing their growth. This phenomenon is normal during limited supply of water, and hence it serves as a survival mechanism (Zhu, 2002). Plant growth and development decreases as a result of deprived root growth, with diminishing leaf-surface traits (shape, form, leaf pubescence and leaf color), which influences the radiation effect on the leaf canopy, delay in rate of normal plant senescence as it approaches maturity, and impediment of stem reserves (Blum, 2011). An increasing number of studies report early morpho-physiological changes in rice after exposure to drought stress. Drought stress leads to reduction in growth and development of rice (Tripathy *et al.,* 2000; Manikavelu *et al.,* 2006). Cell growth is severely impaired due to the reduction in turgor pressure under stress (Taiz and Zeiger, 2006). Drought stress affects both elongation as well as expansion growth (Shao *et al.,* 2008) and hinder cell enlargement more than cell division (Jaleel *et al.,* 2009). A common untimely effect is the reduction in biomass production (Farooq *et al.,* 2010). Many studies report notable decrease in fresh and dry weights of shoots (Centritto *et al.,* 2009) and roots (Ji *et al.,* 2012) under drought. Reduced fresh shoot and root weights as well as their lengths eventually lead to reduction in the photosynthetic rate of physiology and biochemical processes of rice (Usman *et al.,* 2013).

#### 1.2.3.1 Leaf traits affected by drought

One of the acclimation responses of a plant is leaf rolling. It is used as an important criterion for scoring drought tolerance. Many species can reduce the quantity of radiation that they

intercept when suffering from drought stress either by leaf rolling. This is a common stress response that occurs in many grasses and cereals such as rice (*Oryza sativa*) (Matthews *et al.,* 1990).

By rolling its leaves, there are two possible ways in which a plant in a dry environment can benefit. First, by reducing the effective leaf area damage caused by increased leaf temperature resulting from high levels of solar radiation incident on leaf surfaces can be minimized, so that less radiation is intercepted by leaf tissue (Begg, 1980). Second, by leaf rolling transpiration rates can be drastically minimized through the formation of a micro climate having both higher humidity and boundary layer resistance near the leaf surfaces, thereby conserving scarce water resources (Oppenheimer, 1960).

#### 1.2.3.2 Leaf Rolling and Stomatal Conductance

Stomatal closure is a common response to drought stress; it reduces fluxes of both $CO_2$ and water vapor (McCree & Richardson, 1987). With leaf rolling, transpiration varies among different plant species and is dependent on distribution of stomata and the degree and pattern of stomatal opening at low values of $\Psi_w$ in rolled leaves. For instance, in rolled leaves of *Andropogon gerardii* and *Spartina pectinata,* adaxial stomata are closed (Heckathorn & Delucia, 1991) whereas they remain partially open in rolled leaves of *Oryza sativa* (O'Toole & Cruz, 1980). Open stomata with leaf rolling have an advantage compared with total stomatal closure. Instead of closing the stomata in response to stress, rolling can rather be used to adjust the microclimate surrounding the leaf surfaces, allowing water loss to be tuned and photosynthesis and growth to continue (Matthews *et al.,* 1990). Diverse environmental factors influence water loss rates such as temperature, humidity, and wind speed which affect stomatal responses (Aphalo & Jarvis, 1993). Drying of soil also brings about severe reduction in stomatal conductance ($g_s$), which is the rate at which water vapor evaporates from leaf surface via the stomata (Fort *et al.,* 1997). Stomatal conductance and the net $CO_2$ assimilation rate are significantly reduced in unirrigated *Sorghum* lines, in which leaf rolling prevails as compared with irrigated lines (Corlett *et al.,* 1994). In the same manner, although non stressed *Ctenanthe setosa* plants have very low $g_s$ in contrast with other plant species, the $g_s$ value in rolled leaves is approximately one-twentieth as compared in unrolled leaves. Stomata progressively close as drought progresses, and is followed by

parallel reduction of net photosynthesis. Nevertheless, stomatal conductance is not only determined by soil water availability alone, but also by a complex interaction of internal and external to the leaf surface (Medrano *et al.,* 2002).

#### 1.2.3.3 Effect of drought on root traits

For increasing yield in crop plants under water stress, root traits have been claimed to be important. In rice, structure and development of root system primarily determines crop function under drought. Under mild drought stress, the root growth usually sustains while shoot growth is restricted. This is because of the fact that adjustment like, re-establishment of water potential gradient through osmotic adjustment and rise in loosening ability of the cell wall, lead roots to resume growth under low water potential. On the other hand, in leaves there is no such mechanism which leads to marked growth inhibition (Hsiao and Xu, 2000). Root dry mass and length have been reported as good predictor of rice yield under drought (Fageria and Moreira, 2011; Feng *et al.,* 2012).

Extensive investigations on rice roots have led to identification of many root traits that provide drought resistance. Rice genotypes with deep, coarse root system with a higher branching and penetration ability as well as higher root to shoot ratio are reported as component traits of drought resistance (Wang and Yamauchi, 2006; Gowda *et al.,* 2011). Coarse roots have a direct role in drought resistance since roots with larger diameter are related to penetration ability (Clark *et al.,* 2008) and branching, in addition they have bigger xylem vessel radii and lower axial resistance to water flux (Yambao *et al.,* 1992). Ability of deeper root growth and large xylem diameters in deep roots may help largely in root acquisition of water when ample water at depth is available. Small xylem diameters in targeted seminal roots save water deep in the soil for later use during crop maturation. Henry *et al.,* (2012) suggested that reduced xylem-sap bleeding rates from roots, more coherent hydraulic conductivity with variation in soil moisture levels, more reactivity of root anatomy to drought, and higher levels of aquaporin expression are component traits for drought resistance in rice. Trait like xylem pit anatomy that leads to less leaky xylem also promotes plant productivity in water-limited environments without adversely affecting yield under adequate water conditions (Comas *et al.,* 2013).Hence it makes lot of sense for plant scientists for a better understanding of the root physiology under drought thus enabling further insight of important traits that might influence crop productivity under stress and

hence contributing towards selection and development of drought resistant varieties, and thereby improving yield and promoting global food security.

#### 1.2.3.4 Drought and Water Use Efficiency (WUE)

Water Use Efficiency (WUE) is defined as the ratio between dry matter produced and water consumed, measured either at whole-plant level or leaf area (Monclus *et al.,* 2005). Abbate *et al.* (2004) showed that in wheat, WUE under limited water supply was greater than in well-watered conditions. They suggested a correlation exists between higher WUE with stomatal closure to reduce the transpiration. Similarly, in another study conducted on clover (*Trifolium alexandrinum*), WUE was shown to be increased due to lower water loss under drought stress, primarily by decreased transpiration rate and leaf area (Lazaridou and Koutroubas, 2004). Also, in *Pinus ponderosa* and *Artemisia tridentata,* WUE was not reduced due to drought stress but it rather increased, mainly due to a rapid reduction in stomatal conductance with increasing water deficit (DeLucia et al., 1989). Lazaridou *et al.* (2003) further reported that leucern (*Medicago sativa*) cultivated under water deficit conditions had higher WUE than that under irrigated conditions, for the same leaf water potential. However, early season drought stress in potato significantly reduced the water-use efficiency as it became evident through reduction in growth and biomass accumulation (Costa *et al.,* 1997). Hence from above mentioned instances, it is well evident that drought tolerant species maintain higher WUE by reducing the water loss from their leaf surfaces. However, in cases when over-all plant growth was hindered to a much greater extent, WUE also reduced significantly.

### 1.3 Impact of Drought on Agriculture

Most detrimental effect of drought stress on crop plants is reduction in yield, as reported in rice (Brevedan and Egli, 2003), wheat (*Triticum aestivum*, Cabuslay *et al*., 2002), soybean (*Glycine max*,Kirigwi *et al*., 2004), and chickpea (*Cicer aerietum*,Khanna-Chopra and Khanna-Chopra, 2004). Numerous United States Department of Agriculture (USDA) reports have recognized drought as the most frequent yield-reducing factor in arid and semiarid regions, although water deficit may occur even in high rainfall areas (Vamerali *et al*., 2003). Production of cereals and pulses in India was also reduced by about 30% in 1971 due to

prevailing drought situation (Swindale and Bidinger, 1981). The USDA also reported that frequent droughts in years 1980, 1983, and 1988 significantly led to a decrease in maize and soybean yields in USA (Taiz and Zeiger, 1998). Similarly, a heat and drought wave of 2003 caused significant reductions in net primary productivity and decrease in maize yield in both Eastern and Western Europe (Ciais *et al*., 2005). This information is enough to present drought as a potential source of disaster, especially as it affects almost every part of the world; and crops, such as rice, that feed much of the world's population are easily affected by drought.

Estimates show that drought individually lead to 17% potential yield loss in major crops when compared to other abiotic stresses (Ashraf *et al*., 2008). For example Bray *et al*., (2000) estimated that in annual crops 51-82 % yield reduction could be attributed to erratic and insufficient rainfall. Similarly, 24 million tons of maize is destroyed yearly in the tropics due to drought (Heisey and Edmeades, 1999).

### 1.4 Rice as a Model Crop: Taxonomy, origin and cultivation

Rice (*Oryza sativa* L.) is the staple food for more than half of the world's population and hence is the most important crop (Todaka *et al.,* 2012). It supplies 20% of the world's dietary energy needs. It is also a good source of thiamine, riboflavin, niacin and dietary fibre (FAO, 2004).

It belongs to the genus *Oryza* within the Poaceae family. Amongst about 20 *Oryza* species, only two species (*O. sativa* and *O. glaberrima*) are cultivated. *O. sativa* (Asian rice) comprises the *indica* and *japonica* types. *Oryza rufipogon sensustricto* and *Oryza nivara* are the wild progenitors of Asian rice, which are thought to be native of South and Southeastern Asia-extending northwards into Southern China (Fuller 2011), whereas *O. glaberrima* (African rice) originated from the inland delta of the Niger river (Wopereis, 2009).

Rice has been cultivated for more than 7,000 years (Yunfei *et al.,* 2007). It is grown in more than a hundred countries, with a total harvested area of approximately 158 million hectares, producing more than 700 million tons annually (IRRI, 2014). Since rice is a semi aquatic plant, its production is water intensive (Wassmann *et al.*, 2009). Almost 50% of the land used for

rice production is irrigated-34% of total rice cropped area is rainfed lowlands, 9% is rainfed uplands, and 7% flooded systems. Alone irrigated rice contributes 75% of the global rice production (IRRI, 2007).

More than 90% of the world's rice is grown and consumed in Asia, where 60% of the world's population lives. Rapid increase in human population throughout the world is demanding a corresponding increase in grain yield (Liang *et al.,* 2010) and there is need to increase production by 50% till 2025 in order to feed this increasing population (Khush, 2001). For rice consuming countries there is need to produce 40%more rice by 2030 (Zhu *et al.,* 2010). To achieve this ambitious goal of new improved rice varieties with better agronomic and physiological traits such as stress tolerance etc. should be developed.

### 1.4.1 Impact of drought stress on rice

Globally, nearly 80 million hectare of irrigated lowland yield 75% and similarly about 60 million hectare of rainfed lowlands leads to about 20% of the rice production. Rice requires more water as compared to other crops, on an average about 2,500 liters of water is required to produce roughly 1 kg of rice (Bouman, 2009). Irrigated rice requires an estimated 34-43% of the world's total irrigation water. Water requirement for world agriculture is becoming increasingly scarce day by day due to uneven and uncertain rainfall, limiting groundwater resources, increased level of salts in soil solution and diversion of fresh water resources to match urban and industrial requirement. In the future, water availability might be more severely affected due to ongoing changes in global climate and melting glaciers. Because of its semi-aquatic ancestry, rice is extremely sensitive to water shortage.

Drought is the most disastrous form of stress among abiotic stresses and it could lead to decrease in the yield by 15-50% depending on the vigor and period of stress in rice (Srividya *et al*., 2011). The global reduction in rice production due to drought stress averages 18 million tons annually (Lakshmi *et al*., 2012). Rice is sensitive to drought stress but during reproductive growth even moderate stress can result in drastic reduction in grain yield (Venu Prasad *et al.,* 2008). In the eastern states of India viz., Jharkhand, Orissa and Chhattisgarh, 40% of the total rice production is affected by intense droughts valued at $650 million (Pandey *et al.,* 2005) and poorest rice farmers are severely affected in these areas. In rainfed

areas, high yielding semi-dwarf rice varieties are not widely cultivated because of their poor adaptability to more stressful rainfed conditions (Pandey *et al*., 2000). Drought mitigation, through development of drought resistant varieties with higher yields adapted for water-limiting environments will be a key for improvement of rice production and thus will ensure food security to 3 billion people in Asia. The development of such varieties requires a good knowledge of the physiological mechanisms and the genetic control of the traits contributing in drought resistance.

Crop yield is dependent on specific climate conditions and is highly influenced by climate variations. Change in rice yield globally is shown in Fig. 1. The overall rice yield variation due to climate variability over the last three decades was estimated by Ray *et al*., (2015). It was concluded that about 53% of rice harvesting regions are endangered by the influence of climate variability on yield at the rate of about 0.1 ton/ $hm^2$ per year and nearly 32% of change in rice yield is explained by year-to-year global climate variability (Fig. 2). With the worldwide reduction of water supplies for agriculture, the efforts to improve drought adaptation of rice and to screen resistant varieties are becoming increasingly important. The uncertainty of drought patterns and the degree of complexity of the response mechanism involved have made it difficult to characterize the component traits needed for improved drought performance, thereby limiting crop improvement to enhance drought resistance (Serraj *et al*., 2009).

**1.5 Why drought tolerant crops are required**

Apparently, the detrimental effects of drought stress disable farmers' ability to produce high yield crops worldwide. Due to global climate change, predictions forecast an increase in the frequency and intensity of drought and its affected areas with arise in temperatures (Y Li *et al.*, 2009; Dai, 2010). Likewise, rate of reduction in yield for major crops (barley, maize, rice, sorghum, soya bean and wheat) will increase as much as 50% in 2050 and almost 90% in 2100 (Gornall *et al.,* 2010).

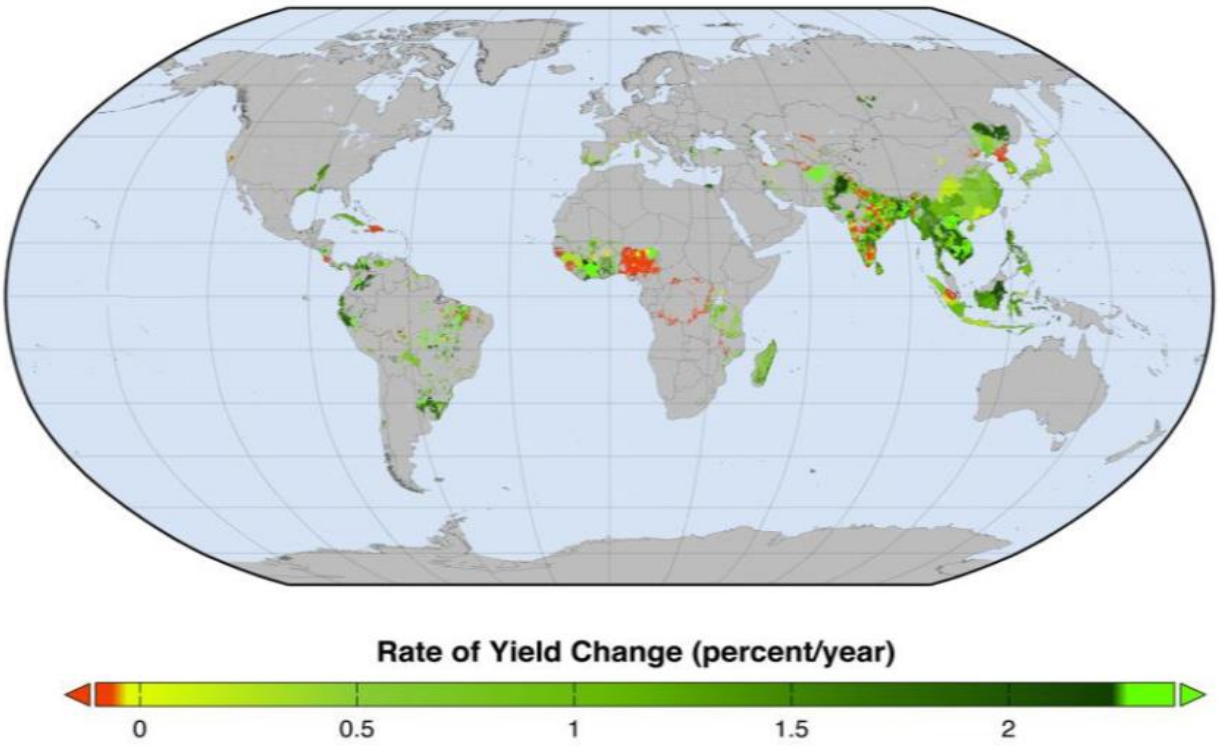



**Figure 1. Global map showing percentage rate of change in rice yield (Ray *et al.,* 2013).**
Red areas show where yields are declining whereas the green areas show where rates of yield increase.

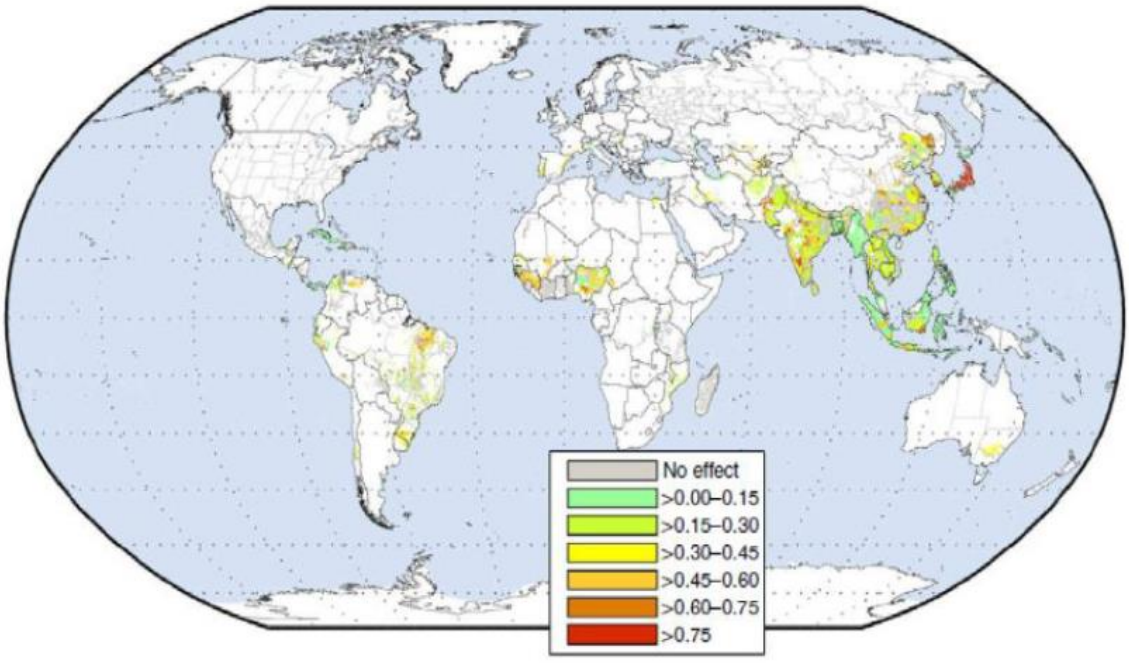



**Figure 2. Global rice yield variability due to climate variability over the last three decades (Ray *et al.,* 2015).**

Based on different models, estimates predict that increase in global drought has been overrated and that there has been small change in drought over the past 60 years (Sheffield *et al.*, 2012). Hence, efforts targeted at improving agricultural adaptation to climate change undoubtly favor some crops and regions over others (Lobell *et al*., 2008). Without adequate adaptation measures, statistical crop models and climate change projections reveal that South Asia and Southern Africa are the probable two regions that will tend to undergo negative impacts on several crops, these regions are important to large food-insecure human populations (Lobell *et al.,* 2008).

By rigorously challenging the capacity to feed increasing human population which is expected to peak at 9 billion by 2050, drought is an important determinant of world food security (Borlaug, 2007; FAO, 2006, 2009, 2011). A significant increase in this population is expected to be contributed by developing countries (FAO, 2009). Predictions indicated that agricultural production and food sufficiency in many African countries would be affected, thereby affecting food security and aggravate malnutrition (Müller *et al.,* 2010). Around 200 million undernourished people live in sub-Saharan Africa (Rosenthal and Ort, 2012) and additionally 480 million African residents are projected to live in areas of scarce water supply by 2025 (UNFCC, 2006).

Breeding or development of crop cultivars that can prevent drought associated hazards is therefore imperative. This would ensure sustainable increase in food production in drought-prone or marginal areas and to feed increasing human population (Baulcombe, 2010). Interpreting the mechanisms of drought tolerance and breeding drought resistant crop plants has been a key goal of crop breeders and plant scientists (Xiong *et al.,* 2006). However, improving the drought tolerance trait has been a difficult task for breeders worldwide (Pray *et al.,* 2011). The reason being drought tolerance is a quantitative trait. There is insufficient understanding of specific traits linked to drought tolerance (Xiong *et al.,* 2006). In defiance of these, considerable efforts have been made in the isolation and functional analysis of genes contributing to yield and abiotic stress tolerance (Takeda and Matsuoka, 2008). Advantageous methods are being developed for identifying additional genes and variants of interest and implementing in practical crop improvement.

**1.6 Drought responses in Plants: Rice as an example**

Drought is one of the major abiotic stresses that limit rice production and yield stability (Lanceras *et al.,* 2004). Different physio-biochemical processes at cell and organism level are known to be associated with drought adaptation mechanisms which have been reported to be complex phenomena (Tripathy *et al*., 2000).

Drought responses in rice expressed by roots, shoots and leaves depend on the timing of stress during plant growth (early, vegetative, intermittent or terminal drought), crop growth stage (seedling, vegetative or reproductive), drought severity level (mild or severe), edaphic properties and the target environment (Fukai and Cooper, 1995).

During water scarcity, development of a deep root system capable to extract water from deeper soil layers (Gouda *et al.,* 2012), may be a rice plant response under drought avoidance (Fukai and Cooper, 1995). Drought escape however is achieved by short growth duration genotypes that avoid the reproductive or terminal drought (Bing *et al.,* 2006). Physiological mechanisms such as osmotic adjustment (Wei *et al.,* 2014), or stomatal conductance (Price *et al.,* 2002), along with biomass production and drought response index (DRI) are important traits for adaptation of rice to drought-prone sites (Xangsayasane *et al.,* 2014). Moreover, rice lines with better adaptation to drought responded with high levels of chlorophyll, soluble sugars and proline, while their malondialdehyde content is lower than in susceptible plants (Wei *et al.,* 2014).

Cytochrome P450 catalyzes many enzymatic reactions for various kinds of substrates, i.e., an oxidative, peroxidative, and reductive metabolism of endogenous and xenobiotic substrate. Specifically, plant P450 participates in various biochemical pathways for the synthesis of plant products including phenylpropanoids, alkaloids, terpenoids, lipids, cyanogenic glycosides and glucosinolates (Chapple, 1989).

Tamiru *et al.,* (2015) reported the characterization of dss1, a rice mutant showing dwarfism and reduced grain size. The dss1 phenotype is due to the effect of a non-synonymous point mutation recognized in DSS1, which is member of a P450 gene cluster located on rice chromosome 3 and corresponds to the previously reported CYP96B4/SD37 gene. (Zhang *et*

*al*., 2014) Hormone profiling showed that the accumulation of abscisic acid (ABA) and ABA metabolites, as well as significant decrease in GA19 and GA53 levels, precursors of the bioactive GA1 (Gibberellic Acid1) , in the mutant. Cytokinin and auxin contents in dss1 were not significantly different from wild-type plants. Consistent with the accumulation of ABA and metabolites, germination and early growth was also delayed in dss1, and this mutant also showed an enhanced tolerance to drought. Moreover, expression patterns of members of the DSS1/CYP96B gene cluster were regulated by drought stress and exogenous ABA. Apart from that, RNA-seq-based transcriptome profiling revealed that cell wall-related genes and genes involved in lipid metabolism were up- and down-regulated in dss1, respectively, in addition to others. All in together, these findings indicates that DSS1 mediates growth and drought stress responses in rice by playing a role in GA/ABA balance, and it is likely involved in lipid metabolism as well.

### 1.6.1 Biochemical, genomic & proteomic responses associated with drought tolerance

Biochemical studies have revealed that sugars (for example - raffinose family oligosaccharides (RFO), sucrose, sorbitol, and mannitol), amino acids (e.g., proline), and amines (e.g., glycine, betaine) accumulate under drought stress in different plant species (Seki *et al*., 2007). As soil becomes water deficit, its water potential becomes more negative. Deposition of solutes (osmolytes) in plant tissues leads to more negative water potentials, allowing them to retain water and avoid reductions in turgor. This drought-tolerating mechanism is known as osmotic adjustment (OA). By contributing in this way to maintain turgor pressure, osmolytes act as protectants for plants subjected to low water potential (Pandey *et al*., 2004). OA helps to maintain cell turgor, which permits cell enlargement and plant growth during water stress; and also allow stomata to remain at least partially open and CO2 assimilation to continue at water potentials that would be otherwise prohibited (Alves and Setter, 2004). OA by osmolytes has been shown to be a reason of improved productivity in wheat (Flower and Ludlow, 1987), sorghum (Sorghum bicolor), barley (Hordeum vulgare), and rice (Lanceras *et al*., 2004). In addition to acting as osmolytes to avoid decrease in turgor for a longer period of time, some of the accumulated solutes are thought to be active in stress-protective functions as free radical scavengers and stabilization of macromolecules during drought (Seki *et al*., 2007).

In many plants that are known to adapt to drought stress, a set of genes are transcriptionally activated, leading to accumulation of new proteins in seeds and vegetative organs and leads to better tolerance to drought. Proteins termed LEA (Late Embryonic Abundant), which were first reported in cotton (Gossypium hirsutum), are a set of proteins that accumulate in embryos late in seed development (Xu *et al.,* 1996) where they are associated in countering desiccation tolerance in maturing seeds. These proteins are also reported to be found in vegetative tissues in response to exogenous ABA application, as well as osmotic and dehydration stress (Liang *et al*., 2006). At least six groups of LEA proteins have been categorized by virtue of the similarity in their constituent amino acid sequences; and group 2, also known as the dehydrins, consists of proteins that are induced by dehydration-related stresses such as osmotic stress and drought (Wang, *et al*., 2006). An association between tolerance to drought stress and these groups of proteins has been observed in some crop plants. In blueberry (Vacinium spp.), the dehydrins were found to be accumulated in response to changes in ABA levels during drought stress (Panta *et al.,* 2001). LEA genes when over-expressed in rice (Xiao *et al.,* 2007), tobacco (Nicotiana tabacum) (Wang *et al.,* 2006), and Arabidopsis thaliana (Figueras *et al*., 2004) led to better drought tolerance in transgenic plants. Although the specific roles of the LEA proteins are not known, it is however clear that they are regulated by ABA and cellular water loss.

### 1.6.2 Role of Phytohormonal balance during drought stress tolerance:

As sessile organisms, plants need to regulate their growth and development in order to respond to external stimuli such as water deficit conditions. These responses are orchestrated by plant growth regulators called as phytohormones. These are the compounds which are synthesized from various biosynthetic pathways and can act either at the site of their origination or can be transported elsewhere in the plant. Overall, plant hormone governs every aspect of plant development and their responses to biotic and abiotic stresses (Jurgens and Wolters, 2009). They often rapidly alter gene expression by inducing or preventing the degradation of transcriptional regulators via the ubiquitin – proteasome system (Estelle and Santner, 2010) . One of the most studied response of plants to abiotic stress, especially drought, is ABA signaling and ABA-responsive genes (Tuteja, 2007).

In addition, there is an increasing evidence to believe that other phytohormones such as jasmonic acid (JA) and its metabolically active derivatives (jasmonates) are also vital signaling molecules which participate in various plant responses during biotic and abiotic stresses (Wasternack 2007; Balbi and Devoto 2008). Moreover, a key position in Jasmonate hormonal network is executed by the plant hormone abscisic acid (ABA). Its role in the control of stomata closure and responses to abiotic stress is well known and extensively studied since decades (Mittler and Blumwald, 2015). Drought stress and/or high salinity results in higher levels of ABA in plants and leads to extensive changes in gene expression (Shinozaki and Yamaguchi-Shinozaki, 2007). ABA can be recognized by receptors in the plasma-membrane of guard cells or within the cytosol (Mittler and Blumwald, 2015). Subsequently signalling is activated and the concentration of Ca2+ in the cytosol tend to increase due to the functioning of calcium channels in the endoplasmic reticulum, which further lead to activation or inhibition of ion channels in the plasma membrane. Because of ion fluxes, water potential in the apoplast reduces and consequently water flows out of the cell resulting in a lower turgor of guard cells and closure of stomata. Because of this central function of ABA in the regulation of stomatal opening and closure and its control over mechanisms involved in stress adaptions it is very important in abiotic stress. However, usually alterations in one hormonal pathway influence the pathways of other hormones and conceivably other hormones, specifically those related to stress and growth responses, contribute to the plant's overall response to the abiotic stress. Currently, one such extensively studied hormonal pathway is that of the jasmonates (Riemann *et al*., 2015).

#### 1.6.2.1 Participation of JA in drought stress responses

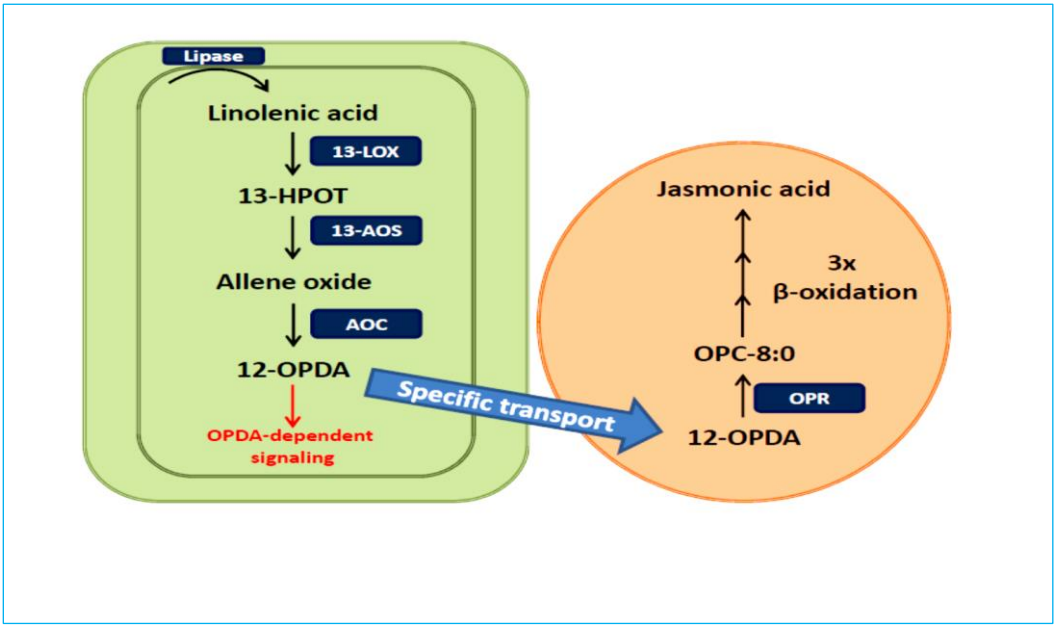
The actual role of JA in drought stress remains controversial. In some investigations JA has been suggested to enhance drought tolerance but in others, it has been reported as a negative regulator that leads to substantial reduction in growth and yield. Basically, the perceived response is dependent on the nature of plant and tissue in query, severity and extent of drought stress and the quantity of JA applied (Kim *et al.,* 2009). Hence, a lot of the controversy might actually exist resulting from the fact that studies were performed under different conditions, e.g. in various developmental stages, tissues, and with different stress regimes (Ismail *et al.,* 2014).

### 1.6.2.2 Regulation of jasmonate biosynthesis

Jasmonates are produced in plants via the octadecanoid pathway (Fig. 3), and are homologous to animal anti-inflammatory prostaglandins in structure and biogenesis (Creelman *et al*, 2002). Its biosynthesis and signaling pathways have been elucidated since the 1980s comprehensively which was reviewed by Wasternack and Hause (2013) on a large scale and big progress in knowledge about the JA pathway has been achieved in rice in the last decade (Dhakarey *et al.,* 2016). JA biosynthesis takes place in two compartments, the chloroplast and the peroxisomes (Figure 3). It is initiated by a lipase which cleaves linolenic acid from a membrane lipid of the chloroplast membrane. Linolenic acid can serve as a substrate for either 9- or 13-LIPOXYGENASES (LOX), but 13-LOXs are required for the biosynthesis of JA. ALLENE OXIDE SYNTHASE (AOS) and ALLENE OXIDE CYCLASE (AOC) convert the product of 13-LOX, (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT), which results in the formation of the intermediate 12-oxo-phytodienoic acid (OPDA). This compound has signaling activity by itself (Taki et al., 2005) , however, for the synthesis of JA it is transported from the chloroplast to the peroxisomes, where it is reduce by an enzyme called OPDA REDUCTASE (OPR) and subsequently goes through several steps of β-oxidation to shorten the side chain. The final product in the peroxisomes is JA, which can freely move to the cytosol.

JA itself is presumably not an active signaling compound, and needs to be conjugated to the amino acid isoleucine (Ile) in a reaction catalyzed by the GH3 enzyme JASMONATE RESISTANT 1 (JAR1) to initiate signaling (Staswick *et al.,* 2002). JA-Ile is recognized by its receptor CORONATINE INSENSITIVE 1 (COI1),(Xie *et al.,* 1998) , an F-box protein forming an SCF complex, which operates as an E3 ubiquitin ligase . The hormone receptor complex recruits JAZ proteins, repressors of JA signaling, and catalyzes their poly-ubiquitination, which marks them for proteolytic degradation in the 26S proteasome (Chini *et al.,* 2007). After that MYC transcription factors are relieved from repression by JAZ proteins, and activate the transcription of early JA responsive genes amongst which are transcripts of the JAZ repressors and further transcription factors. Recently it became also obvious that not just the synthesis of JA-Ile, but that also the inactivation of JA-Ile is a possibility to adjust JA responses. Two major mechanisms to metabolize JA-Ile have been described: one operating

through CYTOCHROME P450s (CYP94 family) (Heitz *et al.,* 2012) another one through AMIDOHYDROLASES such as IAR3 and ILL6 in Arabidopsis (Wideman *et al.,* 2013).




(Adapted from Dhakarey *et al*., 2016)

**Figure 3. Biosynthesis of JA and major enzymes involved.** The biosynthesis occurs in chloroplasts (green) and peroxisomes (brown). In brief, after cleavage of linolenic acid from a membrane lipid it is converted to OPDA in three enzymatic steps. OPDA is a functional signaling compound, but can be transported to peroxisomes specifically where it is further metabolized to JA by the action of OPR and subsequent β-oxidation steps. For further explanations, refer to the text. Abbreviations: 13-LOX: 13-lipoxygenase, 13-HPOT: (13S)-hydroperoxyoctadecatrienoic, 13-AOS: 13-allene oxide synthase, AOC: allene oxide cyclase, 12-OPDA: 12-oxo-phytodienoic acid, OPR: OPDA reductase, OPC-8:0: 3-oxo-2(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid 12-oxo-phytoenoic acid

### 1.7 Drought avoidance and tolerance strategies may be integrated

Drought-avoidance and -tolerance mechanisms were first proposed by Levitt (1972) and since then our understanding of molecular and cellular events involved during drought stress has tremendously increased. It has become clear recently that many of the molecular events triggered by drought do not fit exclusively into avoidance or tolerance categories. For example, accumulated solutes may play a role as osmolytes which facilitates more water uptake by plants and as such constitute a strategy for drought avoidance. At the same time, solutes such as amino acids and sugars may in addition play a protective role against protein and membrane damage. Similarly, dehydrins can act as chaperone-like protective molecules

(Close, 1997) (tolerance mechanism), while their hydrophilin activity (Reyes *et al.,* 2008) support in retaining water (an avoidance mechanism). The role of ABA in ABA-regulated stomatal conductance and root growth (Liu *et al*., 2005) is important in avoidance; but, on the other hand ABA accumulation has been reported leading to synthesis of dehydrins (Xiao and Nassuth, 2006), which have important role to play during drought tolerance.

## 1.8 Various techniques used for plant gene expression studies and their major disadvantages

Plant species whose genomes have been sequenced are model tools to study the function of genes in important biological pathways. These model species include Arabidopsis (The Arabidopsis Genome Initiative, 2000), rice (International Rice Genome Sequencing Program, Goff *et al.,* 2003), poplar (Tuskan *et al.,* 2006), and soybean (DOE Joint Genome Institute, 2008).

Sequencing projects not only produces genomic sequences of model plants of interest but also leads to a large number of expressed sequence tags (EST) for many other crop plant species. With sequencing of the genomes representing model systems as well as crop plants (rice and soybean), plant science have hence entered a new era. However, this field now faces the challenge to provide applications for crop improvement. Many emergent tools now enable a large-scale, parallel, quantitative profiling of molecular states. The high-throughput gene function analysis technologies can identify candidate genes involved in growth and responses to the environment which also include genes involved in drought responses. In the post-genomic era, elucidation of the biological functions of these genes is among the greatest challenge.

DNA microarray technology, which emerged more than a decade ago, has been applied to determine transcript abundance for many or all transcripts in a genome by comparing control and experimental treatments. These microarray data have been utilized for varying number of plant processes related to seed development, wounding responses, pathogen attack and environmental stress responses (Rabanni *et al.,* 2003). Microarrays expression data also lead to new insights into physiological and biochemical pathways of drought tolerance and thus better lead to identification of novel candidate genes that can rapidly advance breeding for drought tolerance. Drought-inducible genes were identified in

Arabidopsis (Seki *et al.,* 2001) on the basis of microarray and RNA gel blot analyses. Similar approaches were also used for identification of dehydration-inducible genes in sorghum (Pratt *et al.,* 2005) and rice (Zhou *et al*., 2007). The major disadvantage of microarrays is that they depend on current genome annotations, which hinders the identification of novel transcripts. Also, there is no specific correlation between mRNA expression levels and their corresponding protein abundances (Gygi *et al.,* 1999). Furthermore, it is impossible to deduce the functional state of a protein purely from expression level of its mRNA.

### 1.8.1 Emergence of Proteomics

Proteins constitute a crucial component of plant stress response machinery since they are important constituents of plant cell structure and metabolism (Kosova *et al.*, 2011). Proteins are the products of genes and they complement the resulting phenotype as they act as direct regulators of the phenotype, *i.e.*, they are the constituents of plant cell structure and actively function in metabolism of all cellular machinery. Proteome- the total of all proteins in a given tissue at a given time in a given condition—is uniquely variable.

Proteome is different from the genome, latter being only one for a given organism but there are infinite proteomes which depend on an organism's growth and developmental stage, plant tissue, and cell type as well as on ambient growth conditions. Additionally, single gene can give rise to various protein products due to various mechanisms of posttranscriptional (alternative RNA splicing, RNA editing, *etc.*) and posttranslational modifications (PTMs—phosphorylation, acetylation, methylation, ubiquitination, myristoylation *etc.*). Hence, the total number of explicit proteins synthesized by a given organism can be several orders higher or lower than the total number of genes encoded by a genome of the organism in question. Considering the great range of proteomes, a plant thus owns a dynamic tool to modulate its response to specific environmental conditions (Kosova *et al.*, 2015).

Thus, proteomics- study of proteome - allow global investigation of structural, functional, abundance, and interactions of proteins at a given time point. As an investigative technique proteomics has an advantage over other "omics" tools since proteins are the key players in majority of cellular events and are the final products of genes involved (Baginsky, 2009). In addition to its capability of complementing transcriptome level changes, proteomics can also detect translational and post-translational regulations, thereby providing new insights into

complex biological phenomena such as abiotic stress responses in plant roots (Salekdeh *et al.,* 2002).

#### 1.8.1.1 Perspectives in Crop Plant Proteomics

After the completion of genome sequencing project of model species such as dicotyledonous plant *Arabidopsis thaliana*, monocotyledonous crop plant rice (*Oryza sativa* ssp. *japonica* and ssp. *indica*), model grass *Brachypodium distachyon*, legume species soybean (*Glycine max*), cereal crops *Zea mays* and *Hordeum vulgare* and with recently released chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome (International Wheat Genome Sequencing Consortium [IWGSC], 2014),efforts have been targeted to link available transcriptomic data to the biological function and functional network of proteins (Hu *et al.,* 2015). Moreover, while dealing with complex and dynamic plant proteomes, it becomes imperative to choose suitable proteomic approaches that lead to identification of proteins and their modification that may contribute to crop improvement (Baginsky, 2009). In the recent years, quantitative proteomic studies with the evolution of high resolution mass spectrometry instruments have been contributing to our better understanding of plant growth, development and plant interactions with the environment (Ghosh and Xu, 2014). This approach is particularly realistic for crop scientists as it may not only contribute for better nutritional value and yield, but also for a better understanding of mechanisms which lead to abiotic stress response of crops (Ghosh and Xu, 2014).

#### 1.8.1.2 Tools and techniques employed for plant proteomic analyses

Development in high-throughput proteomics lead to better understanding of complex biological questions in various species. However, several technical challenges still hinder plant proteomics progress. For example better sample quality is one of the important factors for successful proteomic experiments and is difficult to obtain from plant tissues. An enriched level of oxidative enzymes and proteases in plant tissues make it extremely challenging to extract sta-ble protein mixtures. Moreover, secondary metabolites produced in plant cells often hinder with ensuing protein fractionation and downstream processes. Hence it is rather a difficult to extract fully representative protein classes from plant tissues.

Additional challenges come from the cell wall that is difficult to lyse. Use of TCA (Trichloroacetic acid)-acetone precipitation and phenol extraction method helped to get rid of the above problems to a certain extent (Isaacson *et al*., 2006). However, enhancements to certain experimental conditions are still important taking the consideration of the heterogeneity between species. Additionally, another major limitation to effective extraction of proteins is low protein content in plant cells.

Protein extraction is usually subsequent by protein separation and identification that can be attained with the use of two-dimensional electrophoresis (2-DE) (Wittmann-Liebold *et al.,* 2006) or liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) (Fournier *et al.,* 2007). Advantages of gel based separation techniques have been debated (Gygi *et al.,* 2000) though when compared to the LC-based shotgun approach, both separation strategies are being widely employed carrying their own advantages and disadvantages. Gel based approaches are widely employed for their ease of use, reproducibility, wide molecular weight coverage, and identification of post-translational modifications. However, attentive manual editing is necessary in order to obtain high precision specifically for comparative proteomics. Moreover, narrow pI range coverage and lack of ability to identify low abundant proteins restricted the use of this technique for broad protein mapping (Gygi *et al.,* 2000). Protein spots acquired after separation on a 2D gel are followed by trypsin digestion leading into peptides for subsequent protein identifications. On the other hand, LC-based separation approach requires digestion prior to separation in majority of the cases. This separation system covers a wide molecular weight ranging across identification of low abundant proteins (Fournier *et al.*, 2007).

Protein recognition followed by separation has mainly progressed with the developments in mass spectrometry (MS). It started firstly with the advances in soft ionization methods such as matrix assisted laser desorption ionization (MALDI) (Tanaka *et al.,*1988) or electrospray ionization (ESI) (Yamashita and Fenn, 1984) and secondly peptide fragmentation by collision-induced dissociation (CID) in tandem MS (Stephenson and McLuckey, 1998) lead to excellent coverage. Peptides recognized through MS and MS/MS are searched against specific protein database to obtain a list of proteins. Recent developments in identification of qualitative changes like post-translational modifications permit making difference between identical peptide mass and its altered variants which are critical from biological perspective. With the advent of LC-MS based tagging approaches such as isotope-coded affinity tags (ICAT) (Gygi *et*

*al.,* 1999a), stable isotope labeling by amino acids in cell culture (SILAC) (Ibarrola *et al*., 2004), isobaric tags for relative and absolute quantitation (iTRAQ) (Ghosh *et al.,* 2011, 2013),Tandem Mass Tag (TMT) (Daylon *et al*., 2012) helped to investigate this field by relatively quantifying proteins or peptides at a global level. Introduction of statistically robust label free quantitative approach is also helping quantitative proteomics research to investigate large number of clinical samples (Wu *et al.,* 2006). Therefore, with the available as well as ongoing developments in the MS field, proteomics is expected to provide us improved ways to excavate biological information.

#### 1.8.1.2.1 Quantitative Proteomic: MS based strategies for relative and absolute quantification

As discussed before, genome and transcriptome expression profiling do not accurately relate with proteome complexity (Maier, 2009), the direct and targeted measurement of global protein expression levels alone, better known as quantitative proteomics, can provide valuable information on biological processes in crop plants and hence could pave a way for crop improvement (Vanderschuren *et al*., 2013). In quantitative proteomics mainly protein separation is coupled with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) leading to identification of protein species. In large scale proteomic studies, relative amounts - instead of absolute amounts - of the identified peptides or proteins are quantified, achieved by comparison of the same samples representing different conditions (Bantscheff M.*et al.,* 2007). Relative quantification which is traditionally performed by 2-DE separation methods subsequently followed by staining and image analysis leads to visible differences in gel patterns through the differential staining comparison (Braisted *et al*., 2008). However, this cumbersome and sequential protocol is difficult to automate and suffers from high level of sensitivity and robustness (Abdallah *et al*., 2012).With the evolvement of MS-based proteomics, a relatively new toolbox has rendered available for quantitative analysis. Through shotgun proteomics(bottom-upstrategy) complex peptide fractions produced after protein proteolytic digestion can be better resolved using various fractionation strategies, which render high-throughput analyses of the proteome of an organelle and provide an overview of the major protein constituents (Liu H. *et al.,* 2004).

#### 1.8.1.2.2 Relative and absolute quantitation strategies: When and Why?

Obviously, absolute quantitation would appear to be an ideal strategy compared to relative quantitation, reason being that the absolute peptide values from various samples could also be compared to identify relative protein changes. Relative proteomic quantitation is used very often as compared to absolute quantitation because expensive reagents and time-consuming assay development and optimization are required for the absolute quantitation of each protein of interest.

Experimental bias can affect the reason to choose relative or absolute quantitation protocols. One main source of bias is the mass spectrometer itself, which has a finite capacity to analyze and detect low-abundance peptides in samples with a high dynamic range. Moreover, the limited duty cycle of mass spectrometers impede the number of collisions per unit of time, which may lead to an event of under sampling of complex proteomic samples (Prakash *et al*., 2007). Variation in sample preparation between experiments or individual samples in single experiments could be additional source of bias. The greater the number of steps between labeling and sample combination- risk of introducing experimental bias becomes greater. For example, during metabolic labeling, proteins are labeled in live tissues or cells and then the samples are immediately combined. Since all subsequent sample preparation and analysis is performed using the combined samples, metabolic labeling has the least chance of experimental variation (Bantscheff *et al.,* 2007). On the other hand, samples which are individually processed and resolved in label-free quantitation strategies have the major risk of sample variation and experimental bias (Megger *et al.,* 2014).

### 1.8.2 Tandem Mass Tag (TMT)

TMT is a novel MS/MS-based quantitative method using isotopomer labels referred to as "tandem mass tags" (TMT) was recently developed (Thompson *et al.,* 2003). Both techniques share many common features. (i) These reagents exploit *N*-hydroxy succinimide (NHS) chemistry that allows specific tagging of primary amino groups. (ii) They were devised to permit multiplexing of several samples by chemical derivatization with various forms of the same isobaric tag that emerge as single peak in full MS scans. (iii) The release of "daughter ions" in MS/MS analysis (between 126 and 131 Da for TMT) can be utilized for relative quantification. The cysteine reactive TMT (cys TMT) reagents perform selective labelling and

relative quantitation of cysteine-containing peptides from up to six biological samples (Abdallah *et al.,* 2012).

Since TMT tagged peptide pairs are isobaric, they co-elute during chromatographic separations and lead to more accurate quantification than conventional isotope labeling strategies (i.e. ICAT). Additionally, the MS signal of each peptide pair is not split into different peaks because of mass shifts thereby improving the sensitivity in the MS mode (Thompson *et al.,* 2003). A particular benefit of isobaric mass tags is the multiplex abilities and thus increased throughput potential of this strategy. Commercially available isobaric mass tags (e.g., TMT*, iTRAQ*) are available that render the simultaneous analysis of 4, 6 or 8 biological samples. Though the exact tags used are manufacturer dependent- but the basic components of all isobaric mass tag reagents is made up of 1) a mass reporter (tag) that has a unique number of $^{13}C$ substitutions 2) a mass normalizer that has a definite mass that balances the mass of the tag to make mass of all tags equal. Isobaric mass tags also consists a reactive moiety that crosslinks to primary amines or cysteines (depending on the product used). These tags are devised so that the mass tag is cleaved at a specific linker region upon high-energy CID (HCD), giving rise to the different-sized tags that are then quantitatively analyzed by LC-MS/MS (Figure 4) . Isobaric mass tagging has also been adapted for use with protein labeling (similar to ICPL) (Thermofisher Scientific Ltd).

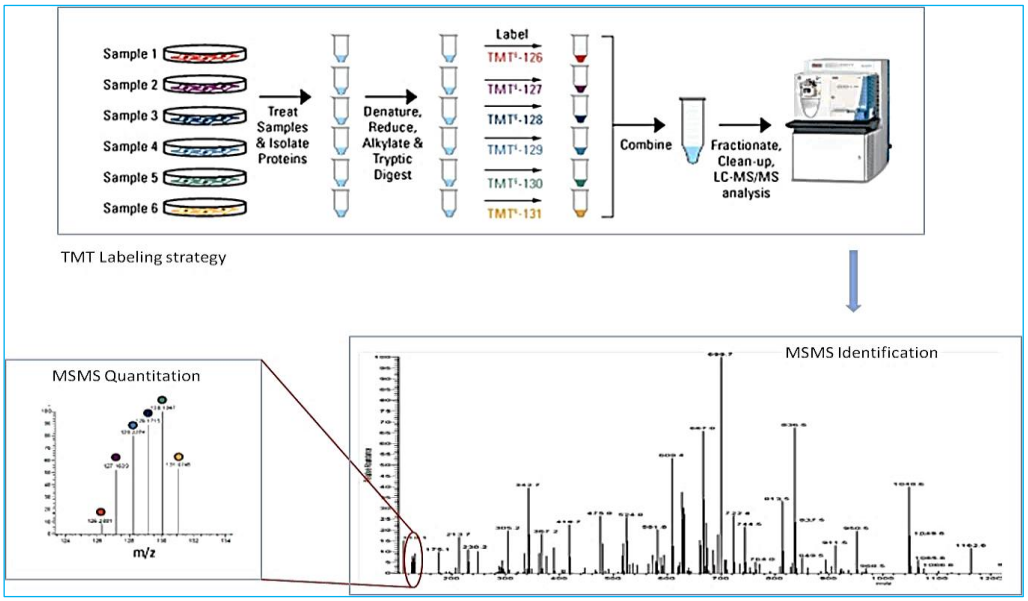



**Figure 4 Strategy for experimental procedure using Thermo Scientific™ TMT 10plex Isobaric Mass Tagging Reagents** Protein isolates from tissues are reduced, alkylated and digested overnight. Samples are labeled with the TMT reagents and then pooled prior to sample fractionation and clean up. Labeled samples are resolved by high-resolution Orbitrap LC-MS/MS prior to data analysis to determine peptides and quantify reporter ion relative abundance. **Source** : Thermo Scientific™

## 1.9 Scope of the Study

Since breeding for drought-tolerant rice is hard to achieve by conventional strategies, including marker-assisted selection - understanding of the molecular mechanisms underlying drought tolerance is therefore needed for successful, knowledge-based crop improvement (Milan *et al.,* 2006). The valuable information gathered, about the role of JA at physiological and molecular level in drought stress, through the use of the mutant approach in this PhD project - could potentially serve as a valuable crude material for plant breeders to develop drought-tolerant crop plant varieties in future. Riemann *et al.,* (2013) successfully isolated and characterized one such JA-deficient mutant called *cpm2*, which was successfully used in this study. *Cpm2* (or *coleoptile photomorphogenesis 2*) has been described as a specific mutant of *ALLENE OXIDE CYCLASE (AOC)*, carrying an 11 bp deletion within the first exon of this gene. Homozygous seedlings of the JA-biosynthesis mutant (*cpm2*) were compared to its corresponding wild type background rice cultivar (Nihonmasari), in drought stressed and well-watered condition, at the level of phenotype, physiology and molecular biology - in order to reveal more extensive knowledge about the involvement of jasmonates in drought response in rice.

A targeted approach has been used by measuring some of the physiologically relevant traits and then by analyzing the root proteome of drought stressed and well-watered wild type and mutant *cpm2* seedlings, in order to understand the possible molecular mechanisms underlying their physiological and molecular response. The proteomic approach has been previously described as quite promising. For instance, in barley it has identified proteins and their corresponding genes associated with metabolism, synthesis of osmoprotectants, and ROS (reactive oxygen species) scavengers (Guo *et al.,* 2009).

The study in this PhD project proceeded in the following four main stages using the genotypes mentioned above:

1. Various physiological parameters related to roots, shoots and at the whole plant level were observed by measuring the root architecture, stomatal conductance, water uptake rate and the Water Use Efficiency (WUE). Shoot ABA levels were also measured in order to relate ABA activity to stomatal conductance of the leaves.

2. Root proteome profiling was performed on roots under well-watered (WW) and severe drought stressed (DS) samples. The TMT (Tandem Mass Tag) technique was used to label the total protein extract obtained from the root tissue. Root proteins differentially expressed between the two genotypes under WW and DS were analyzed by the *MAPMAN* software. The roots were chosen for performing the proteomics because of the widely believed fact that they are the first organs to sense a water shortage (Trachsel *et al.,* 2010). It is also because of this reason, the root system is therefore generally considered as the most important organ with respect to improving crop adaptation to water stress (Vadez, 2014).
3. A number of unique and differentially expressed proteins observed in proteomics data were further considered to be analyzed for their transcript abundances in order to ascertain a co-relation between transcript abundance and their corresponding protein levels.
4. The results obtained from the first three approaches were analyzed and used to draw conclusions regarding the identified proteins involved in the higher drought tolerance of *cpm2*. Furthermore, the results were compared to previously published work on drought tolerance of cereals, and possible mechanisms/roles for the identified proteins were proposed and discussed in order to better relate them with observed physiological data.

## 2. Material and Methods

### 2.1 Plant growth and drought stress conditions for drought phenotyping and stomatal conductance

*Oryza sativa* L. *ssp. japonica cv.* Nihonmasari as the wild type (WT) and the *cpm2,* the mutant generated in the same cultivar (Riemann *et al.*, 2013) were used in this whole study. After dehusking the caryopses, they were surface sterilized by incubating the seeds in 70% ethanol for 1 min and then washed briefly twice with double-distilled water. Then after, the seeds were incubated for 30 min in a sodium hypochlorite solution containing ~5% of active chlorine followed by five washing steps with sterilized double-distilled water. The seeds were sown on 0.5% phytoagar medium (Duchefa, Netherlands) and were kept for 14 d in a culture room (at 25 °C, continuous light of 120 µmol $m^{-2}s^{-1}$). 14 day old seedlings were then transferred to phytochamber (BBC York, Mannheim, Germany) and were left to grow for another 1 week under short-day conditions (10 h light at 28 °C, 280 µmol/$m^2$s, 14 h darkness at 22 °C). Once a week plants were fertilized (Wuxal, "TopN" and "Super" fertilizers, Manna, Ammerbuch-Pfaffingen, Germany). Rice seedlings (aged 21 days) were subjected to drought stress in the phytochamber for 6 days withholding water and then subsequent phenotypical changes were photographed on $7^{th}$ day after imposing stress. Stomatal conductance was measured on youngest fully-expanded attached leaves of each cultivars of same age with 3–5 replicates with a portable-type Porometer (Decagon devices, USA) for 4 days after withholding water and for 2 days after rewatering.

### 2.2 Greenhouse seedling-stage drought studies using mylar tubes for root architecture analysis

Mylar tube experiment was conducted in the glasshouse at the International Rice Research Institute (IRRI), Los Baños, Philippines (Los Banõs, Laguna, 14°10'11.81"N, 121°15'39.22"E). Soil from the IRRI upland farm was dried in a greenhouse, sieved (6mm), steam sterilized, and packed to a bulk density of 1.2g $cm^{-3}$ in 5-cm diameter mylar tubes to a depth of 40cm. All tubes were closed at the bottom with a layer of cotton cloth to allow water flow to the soil, and then inserted inside an outer tube of opaque PVC painted white that had a water-impermeable sealed bottom (Figure 5A). Well grown WT and *cpm2* homozygous seedlings were sown in mylar tubes after growing them for 14 days in a culture room (at 25 °C,

continuous light of 120 µmol $m^{-2}s^{-1}$). Soil moisture treatments of control (100% field capacity), dry-down to 75% of field capacity (75% SMC) and dry-down to 50% of field capacity (50% SMC) were applied. For the control and drought stress (DS) treatments, tubes were soaked for 2 hours, and then allowed to drain overnight before planting. For the 75% and 50% SMC treatment, half of the required water volume was added to the top of the tube and half to the bottom, in order to have a continuous water column in that low-moisture treatment Five replicates per genotype were used for this study. Tubes were weighed three times per week to monitor transpiration, at which time control tubes were watered to maintain 50 % Soil Moisture Content (SMC), 75% SMC and 100% SMC (field capacity) throughout the experiment. The respective target weights for each of the SMC were calculated using stoichiometric methods. Unplanted controls for each treatment were included to monitor evaporation from the soil surface. After harvesting the root material, roots were washed and stored in 75% ethanol until scanning and analysis for root architecture study using *WinRhizo* (Régent Instruments, Quebec, Canada).

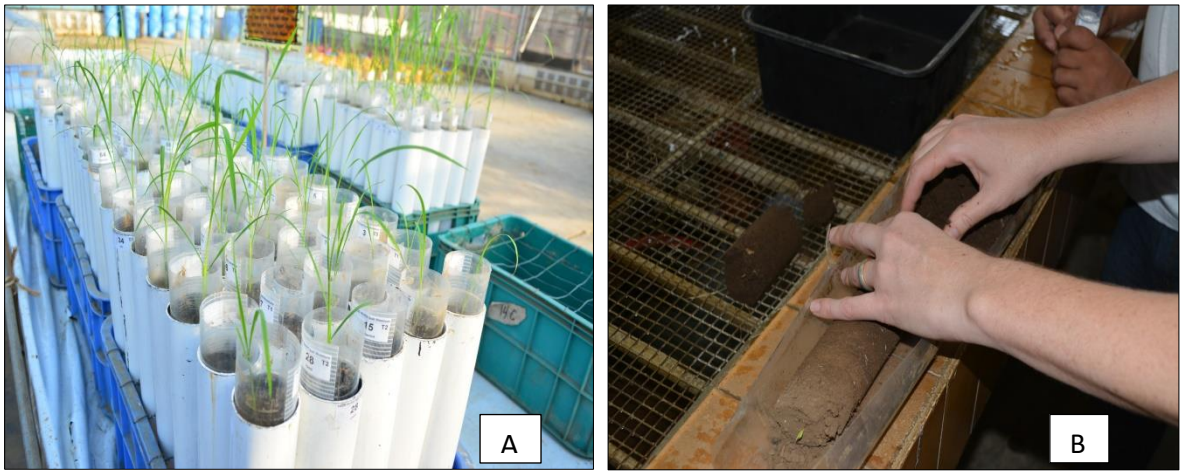



**Figure 5. Root Architecture study was performed using mylar tubes in green house conditions. A.** WT and *cpm2* plants were sown in mylar tubes **B.** Excision of root material and subsequent washing was performed prior to *Winrhizo* measurements.

### 2.3 Experimental setup for the lysimeter study for calculating water use efficiency

Lysimeter experiment was conducted in the glasshouse at the International Rice Research Institute (IRRI), Philippines (Los Banõs, Laguna, 14°10'11.81"N, 121°15'39.22"E) for measuring Water Use Efficiency (WUE). The experiment was performed in lysimeters made up of PVC cylinders 95 cm in height and 20 cm in diameter lined with plastic sheet filled in with soils that was brought from upland and lowland fields at IRRI. Before filling these

cylinders, an upland soil (mollisol) was autoclaved, air-dried and sieved and a lowland soil was made free of debris and maintained saturated. The cylinders were then filled with 25 kg of dry upland soil (bulk density 1.1 g cm–3) that was well compressed manually to a height of 75 cm: 5 L of water was also added into each cylinder to compact the soil furthermore before filling the lowland soil at top. Wet lowland soil was then filled on top of the upland soil, leaving a space of 5 cm at the top of the cylinder. A total of 30 lysimeters were then placed inside a concrete tank (1.35m depth, 3.5m width and 6.8m length) within the glasshouse. Within the lysimeter facility, the cylinders were moved using a cylinder-lifting system that consisted of an electric motor (Shopstar Electric Chain Hoist, Columbus McKinon Corp., Amherst, NY, USA) attached to a custom-built gantry crane that rolls along the top of the cement tank walls (See Figure 6). Each cylinder was lifted one at a time and placed on a weighing balance (KERN SCE-3.0, Kern and Sohn GmbH, Balingen, Germany) and simultaneously imaged with a digital camera (PowerShot G7, Canon, Tokyo, Japan) that was fixed at a distance of 1m from the balance. A black curtain was attached to the moving crane that was positioned behind the balance to provide a black background for the plant images.

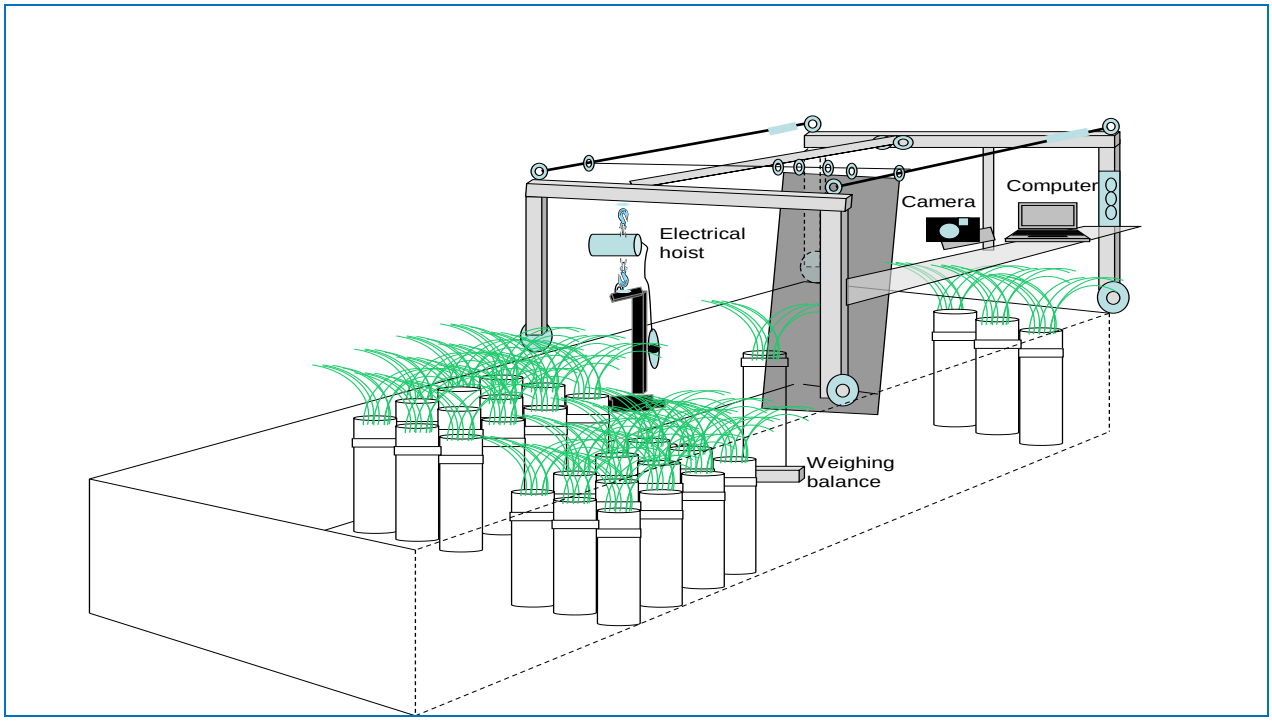



**Figure 6. Illustration of the IRRI lysimeter facility used in this study.** Water uptake was monitored by weighing of lysimeters using an electrical hoist suspended by a custom gantry crane. Lysimeter weights and digital images of shoots were acquired simultaneously to calculate Water Use Efficiency (WUE).

### 2.3.1 Plant growth and their management for calculating water use efficiency using lysimeter

Well grown WT and *cpm2* homozygous seedlings were sown in lysimeters after growing them for 14 days in a culture room (at 25 °C, continuous light of 120 µmol $m^{-2}s^{-1}$). The lysimeters were arranged in an alpha-lattice design and complete fertilizer, NPK (14 : 14 : 14), was applied at a rate of 5.4 g per lysimeter ,1 day after transplanting for proper plant establishment. Plants were top-dressed with 3 g per lysimeter of ammonium sulfate at 24 days after sowing (DAS) and 4 g of ammonium sulfate was later applied at 30 DAS. Thinning was conducted 7 days after transplanting, leaving one plant in each cylinder. Weeds were manually removed regularly. Drought stress was imposed to plants in the drought stress (DS) treatment starting at 32 DAS in the experiment by draining water from the lysimeters through three holes that were drilled near the bottom of the lysimeters and by withholding any further addition of water to those lysimeters. All lysimeters were then well covered with polythene sheets sealed around the base of each plant to minimize direct evaporation, in order to ensure that only water loss from the cylinder by transpiration was measured. All the lysimeters labelled as control were kept well-watered using the target weight which was established by measuring the lysimeters at day 36 and the DS lysimeters were kept unwatered for the rest of the duration of experiment. Experiment was continued for about 4 weeks after draining the lysimeters and the lysimeters were weighed three times a week (Mondays, Wednesdays and Fridays). Well-watered conditions were maintained in the control treatment by adding water as needed every other day.

Digital imaging for plant shoot growth analysis was also performed as described in Kijoji *et al.* (2012) in order to estimate leaf area for the plants growing in all lysimeters. This leaf area was later normalized using *image J* (NIH, USA) with water uptake/day to calculate WUE (Figure 7).


**Figure 7. Calculation of water use efficiency using Lysimeters at IRRI facility.** Digital imaginery for non-destructive monitoring of leaf area for calculating Water Use Efficiency (WUE)

## 2.4 Plant materials, growth and stress conditions for proteomics, ABA measurement and transcript analysis experiments

The sampling material for these experiments were raised in the greenhouse at International Rice Research Institute (IRRI), Philippines (Los Banõs, Laguna, 14°10'11.81"N, 121°15'39.22"E) during the 2014 and 2015 dry season. WT and *cpm2* seedlings were grown in 0.5% phytoagar as described above for 14 days and afterwards well-grown seedlings were transferred to 20 cm pots filled with soil (brought from upland farms of IRRI) and were kept in the greenhouse. All the plants were kept well-watered for another 1 week after which the plants were separated into two categories 'control' and 'stressed' plants. The control plants were kept well-watered and their roots were sampled at day 22 and in parallel rest of the plants were stopped watering for initiating moderate and severe stress condition. For sampling drought stress samples, soil water potential in the drought stressed treatment was monitored by tensiometers (Soil moisture Equipment Corp., CA, USA; one per replicate) and root samples for these stress treatment were harvested when soil moisture content (SMC) reached 30% and 15% respectively for moderate and severe stress and were immediately frozen in liquid nitrogen.

### 2.4.1 Protein extraction, separation, tryptic digestion and TMT labelling

For total protein extraction, frozen rice root samples (100 mg) from two genotypes (WT and *cpm2*) were washed with distilled water to remove soil and other debris. Subsequently, they were pulverized with liquid nitrogen into fine powder to which 0.7 mL of Tris–Cl buffer (pH 8.0) was added ( for overview see figure 8). After subsequent protein extraction using the method as described in Raorane *et al.* (2015a), protein samples were then run through SDS-PAGE under denaturing conditions as per the Laemmli method (Laemmli 1970). Subsequently protein bands were cut and collected from the three independent replicate gels manually and were excised into smaller pieces. These gel pieces were washed twice with 50 µL of 50 % acetonitrile (ACN)/50 % 200 mM ammonium bicarbonate (ABC) for duration of 5 min and were shrunk with 100 % ACN until they appeared white; the gel pieces were then dried for 5 min in a concentrator (miVac, Genevac, UK). Afterwards, the gel pieces were made to rehydrate at room temperature in 15 µL of 50 mM ABC (37°C for 4 min). An equivalent amount (15 µL) of trypsin solution (Promega, USA; 20 ng/µL in 50 mM ABC) was then added, before the gel pieces were incubated at 37°C for at least 16 h. After performing the tryptic digestion, the digests from gel pieces were extracted by using 0.1 % formic acid in 50 % ACN. All of the extracts were then dried in a concentrator. On each liquots, TMT labeling was performed with TMTs with respective reporters of m/ z = 126.1, 127.1, 128.1, 129.1, 130.1, and 131.1 provided by Thomson (Th) in 40.2 µL $CH_3CN$. After subsequent incubation at room temperature, 8 µL hydroxylamine 5 % (w:v) was added in each tube and mixed for 15 min. These aliquots were then subsequently combined, and each pooled sample was vacuum evaporated. The subsequent sample was then dissolved in 1894 µL $H_2O$/TFA 99.9 %/0.1 % prior performing the LC–MS analysis.

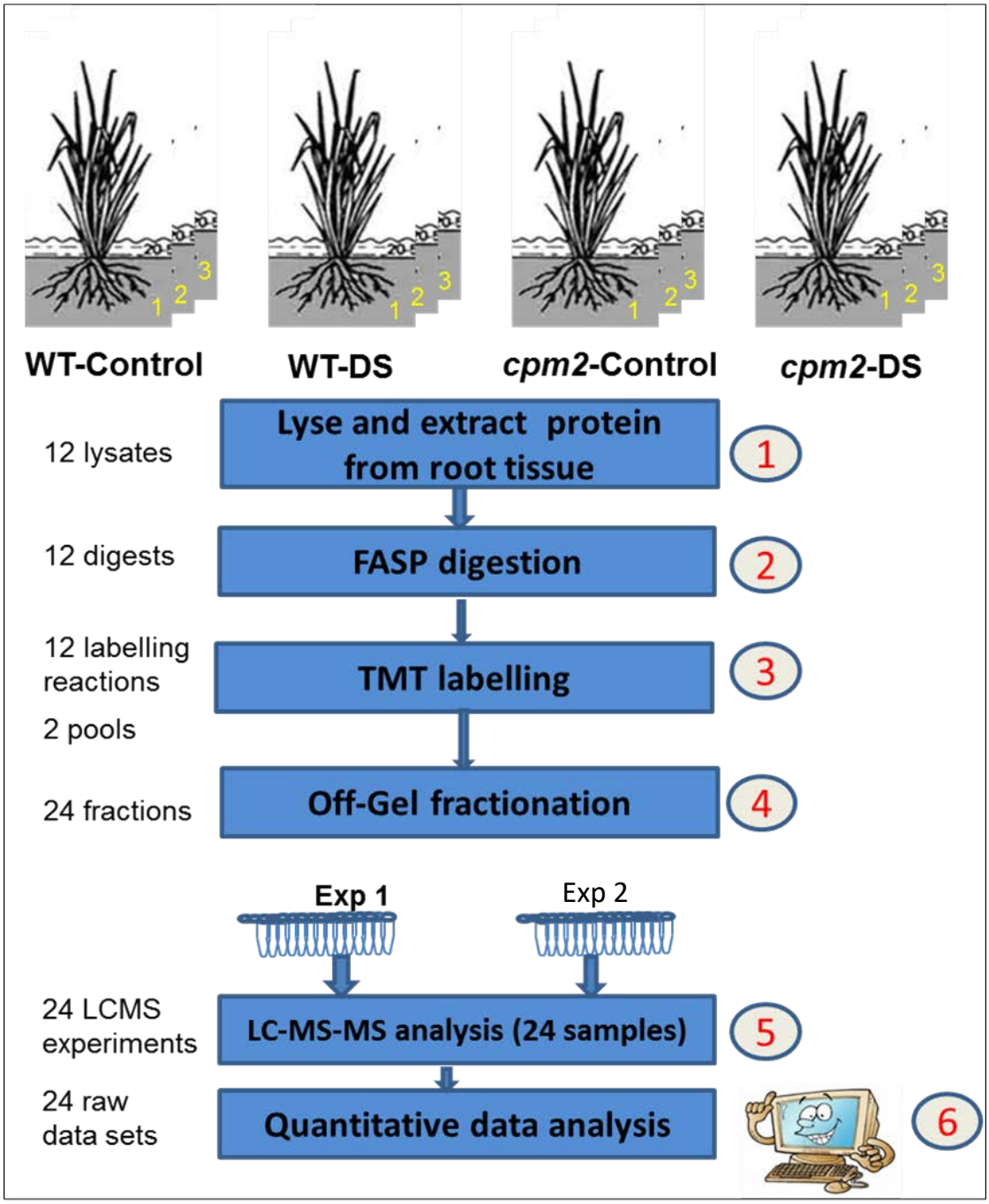



**Figure 8** : **Strategy for TMT labelled LC-MS/MS analysis for comparative proteome analysis of drought stressed rice roots of *cpm2* and corresponding WT**. Samples were collected to extract total proteins. After total protein extraction was digested with trypsin, the peptides were labeled with the TMT reagent and pooled. Pooled peptides were fractioned using the reversed-phase HPLC system, then individual fractions were analyzed using LC-MS/MS. MS raw data were processed using the swissprot protein database. Identified proteins with one or more than one peptide with MASCOT scores greater than 40 were immediately accepted. Single peptides with MASCOT scores less than 40 were deleted from the analysis to avoid false positives. The MSU TIGR v7.0 locus identifiers of the remaining proteins were retrieved using the ID mapping tool in UniProtKB (www.uniprot.org) for input into MAPMAN.

### 2.4.2 Nano-LC–MS/MS analysis

For nano-LC- MS/MS analysis, each digested peptide mixture (5 µL) was introduced into the mass spectrometer through high-performance liquid chromatography by using a 1200 series binary HPLC pump (Agilent, CA, USA) and a FAMOSTM well-plate micro-autosampler (LC Packings). The sample was loaded into a 2cm×75 µm i.d.trap column packed in-house with C18 resin (Magic C18AQ, 5 mm, 200 A˚ ;Michrom, Bioresources, CA, USA) for each analysis. This trap column was connected to an analytical column (11 cm × 75 mm i.d.), and both the columns were rigidly packed in-house with C18 resin (Magic C18AQ, 5 mm, 100 A˚). Mobile phase A was composed of of 0.1 % formic acid, and mobile phase B was composed of 0.1 % formic acid in 100 % ACN. The flow rate was maintained at nearly 250 nL/min under an in-house split flow system. Each reversed-phase step started with 5 % ACN for a duration of 10 min, a gradient of 5–40 % ACN for duration of 75 min, 40–85 % ACN for duration of 5 min, 85 % ACN for duration of 10 min, and then was re-equilibrated with 5 % ACN for a duration of 20 min. Mass spectrometric analysis were carried out with a high performance LTQ XL linear ion trap mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA). A complete-mass scan was performed between m/z 350 and 2000, which was followed by MS/MS scans of the five highest-intensity precursor ions at 35 % relative collision energy. A dynamic exclusion was also enabled with a repeat count of 1, exclusion for duration of 3 min, and with a repeat duration of 30 s.

### 2.4.3 Protein identification

For protein identification strategy, SwissProt protein database 56.8 (release of February 10, 2009) was searched against the acquired MS/MS spectra using the Mascot Daemon version 2.2.2, and the taxonomic category was selected as *Oryza sativa*. For peptide mass tolerance and fragment tolerance, values were set at 2 and 0.5 Da, respectively. The initial search was specified to allow for up to two missed tryptic cleavages. For determining the false positive rates, a Decoy database was performed. The false-positive rates were allowed below 5 % by specifying p-value at 0.025.

### 2.4.4 Functional annotation

Proteins identified with one or more than one peptides with MASCOT scores greater than 40 were immediately accepted. To avoid false positives, Single peptides with MASCOT scores less than 40 were deleted from the analysis. The MSU TIGR v7.0 locus identifiers of the subsequent remaining proteins were retrieved using the ID mapping tool in UniProtKB (www.uniprot.org) for their input into MAPMAN. Finally, a total of 808 proteins, of 272 and 217 unique proteins, with TIGR locus IDs in WT and *cpm2* roots respectively, were used for further functional annotation using MAPMAN. These proteins were mapped on the already available rice-mapping files, and the subsequent mapped proteins were further classified into 24 functional categories based on MAPMAN BINs as described by Thimm *et al.* (2004).

### 2.4.5 Quantitative PCR

Quantitative RT-PCR analysis of a selected set of genes was performed. The proteome analysis suggested candidate genes; those that appeared especially relevant to the JA pathway were assessed at the transcript level in the root tissue of the two genotypes under well-watered and drought conditions. The primers (Supplementary Table 3) were designed using Primer 3 and the rest of the procedures involving RNA extraction; cDNA synthesis and qRT-PCR were carried as described in Raorane *et al* (2015b). OsCyclophilin-2 was used as housekeeping gene for all qRT-PCR studies.

## 2.5 Measurement of endogenous ABA levels in shoot samples

ABA was quantified from shoot samples using a standardized ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS)-based method according to Balcke *et al.* (2012) using [2H6] ABA as internal standard

# 3. Results

The experiments conducted in this study were motivated by the question, which role jasmonate signalling plays for the adaptation to drought stress. Therefore, a comparative study was performed using a rice JA-biosynthesis mutant, *cpm2*, to the wild type (WT) background rice cultivar (Nihonmasari) at the level of morphology, physiology and molecular biology. Surprisingly, the analysis of phenotypic traits and physiological parameters showed that leaves and roots of *cpm2* exhibited less stress symptoms. This allowed assigning the molecular differences, identified by whole-proteome profiling by the tandem-mass tag (TMT) technology on the drought stressed roots of *cpm2* and corresponding WT to adaptive events. The result section is therefore composed of four main subsections:

1. Description of the phenotypical and physiological responses of mutant versus wild type to drought stress.
2. Description of the proteome profiles of mutant versus wild type as identified by TMT proteomics.
3. Assignment of the identified protein to different gene ontologies (so called bins).
4. Cross-connection of these clusters according to functional complexes.

## 3.1. Description of the phenotypical and physiological responses of mutant versus wild type to drought stress.

### 3.1.1 JA-deficient mutant *cpm2* leaves showed less leaf rolling and wilting under drought stress

Plants of WT and *cpm2* grown for four weeks were subjected to drought stress by withholding water in order to observe how JA deficiency affects the drought response phenotypically. After 2 days of withholding water, leaves of *cpm2* appeared to be less sensitive with respect to wilting and leaf rolling and did not roll, while the wildtype leaves were beginning to roll as a response to drought stress (Fig 9A & B). After the end of day 4, WT leaves showed the symptoms of wilting, whereas the mutant leaves remained unfolded and appeared turgescent (Figure 9C & D). Hence, the JA deficient mutant *cpm2* showed less sensitivity to drought during the first few days of stress application. Therefore, it was decided to compare further physiological and molecular parameters in wild type and mutant plants in response to drought stress.

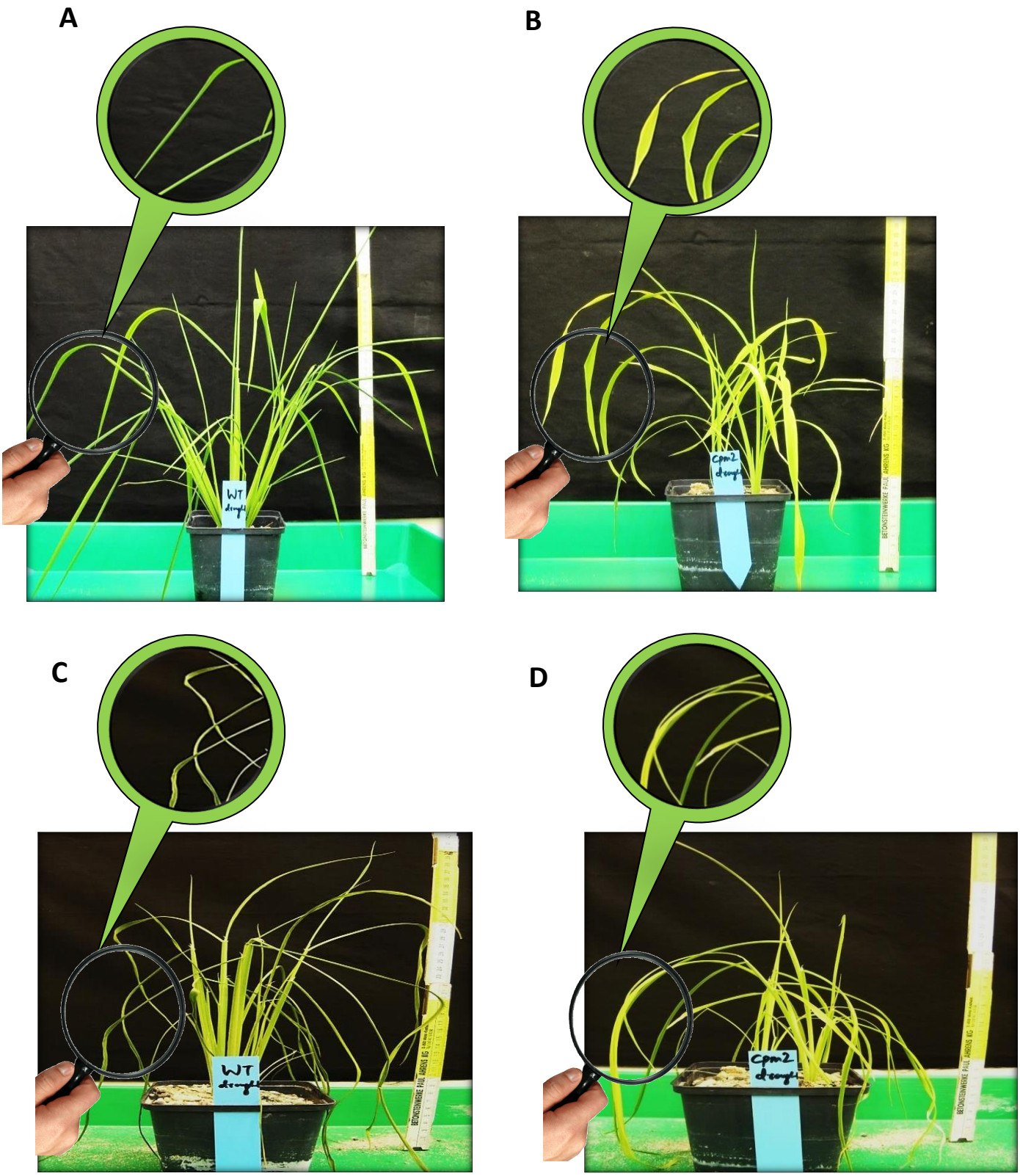
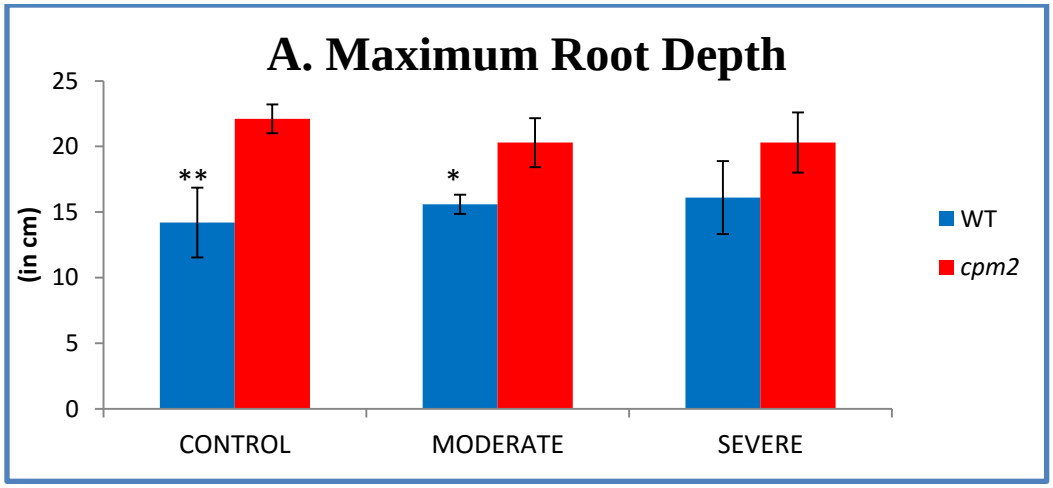


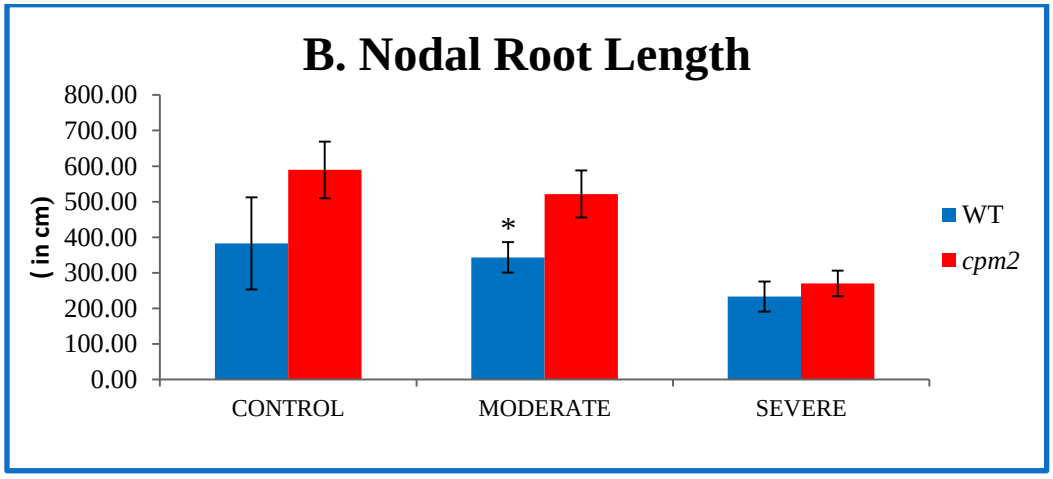

**Figure 9: Phenotypic changes observed in rice seedlings after A) 2 days exposure of drought stress on WT B) 2 days exposure of drought stress on *cpm2* C) 4 days exposure of drought stress on WT D) 4 days exposure of drought stress on *cpm2*.** The 4 week old seedlings of WT and *cpm2* were subjected to drought stress in phytochamber by withholding water and then were photographed subsequently after first appearance of drought stress symptoms. The magnifying glass specifically highlights the leaf area showing drought stress symptoms.

### 3.1.2 The mutant *cpm2* is able to sustain a rich root architecture and good biomass under moderate drought stress

Morpho-physiological measurements in drought studies are crucial for understanding the extent of stress experienced by the plant arising due to the stress treatment and also to provide a framework to interpret the changes on the molecular level described in the sections below. Plants also respond to drought stress in different ways, for example short durations of severe stress often lead to short-term but eventually unsustainable reactions of the "wait and see" type, however long-term stress requires the initiation of more exorbitant avoidance mechanisms that need significant developmental changes. Hence, it becomes crucial to choose such a kind of experimental set-up which can remove the biasness and can provide reproducible results with a degree of robustness (in order to impose appropriate degree of stress). Because of aforesaid reasons, mylar tubes were chosen for carrying out this study (see materials and methods) to perform the experiment under a progressive drought stress condition in a greenhouse at IRRI, Philippines as these conditions together closely mimic the drought conditions prevalent in a rice field.

In order to see the differential effect of drought stress on root growth in the WT and *cpm2*, the root architecture was examined by scanning the harvested root material from the Mylar tubes using the Winrhizo image analysis system. Overall, there were significant differences observed across the treatments where *cpm2* seemed to develop more roots than the wild type and could maintain this feature even under drought.



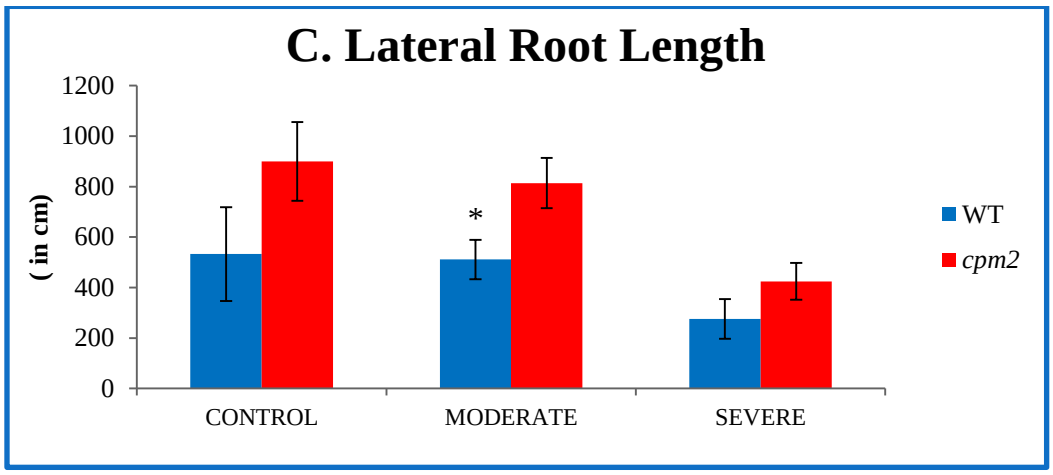




**Figure 10. Differences in root architecture in WT and *cpm2.*** Plants of two genotypes were grown in soils at different moisture contents**.** Significant differences were observed across the treatments in which *cpm2* seemed to be performing better. Values for **A)** Max Root depth **B)** Nodal Root Length **C)** Lateral Root Length are shown here. Data are mean values ± standard error of five replicates. Stars (* & **) denote statistical significance (P ≤ 0.05 & P≤0.01) respectively between the two genotypes in a student's t-test.

Maximum root depth (Mrd) was more in *cpm2* compared to the WT across all the treatments (Figure 10A). However, the WT showed an interesting stress inducible increase in Mrd compared to *cpm2*. Maximum root depth increased for WT as the severity of the stress increased. The mutant *cpm2* showed an adverse effect on Mrd with increasing drought stress. These results indicated that WT roots were capable to increasingly scavenge for water compared to the *cpm2* by showing an increasing response of Mrd towards stress.

The nodal root length was also measured under the three treatments in both the genotypes. Across the treatments, WT showed ~ 40% reduction in nodal root length from control to severe stress condition. On the contrary *cpm2* showed ~50% reduction in the nodal root length from control to severe stress condition (Figure 10B). The lateral root length was also measured across the treatments for the two genotypes. It was established that both the WT and *cpm2* plants showed ~ 51% reduced lateral root growth as the severity of the stress increased. It was also observed that both the nodal and lateral root lengths for the WT and *cpm2* showed marked reduction in severe stress condition than in the moderate stress compared to their respective length at control conditions. (Figure 10 C).

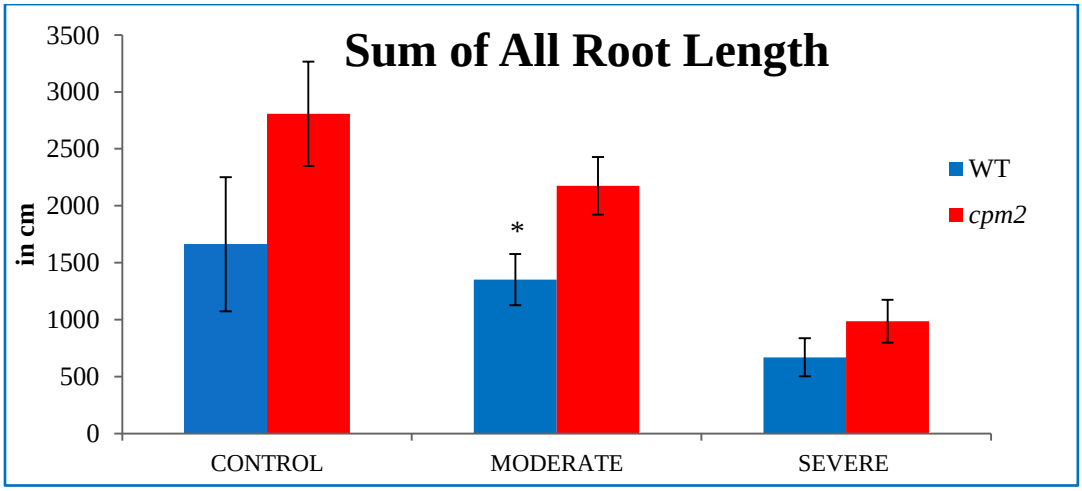



**Figure 11. Sum of All Root Length are shown here.** Plants of two genotypes were grown in soils at different moisture contents**.** Significant differences were observed across the treatments in which *cpm2* seemed to be performing better. Data are mean values ± standard error of five replicates. Stars denote statistical significance ($P \leq 0.05$) between genotypes in a student's t-test.

The sum of all root types was calculated by summarizing overall root architecture. This indicated that in *cpm2*, the three different treatments showed much higher root length than the WT control (Figure 11). Thus these root morphological characterization suggested that although the effect of drought stress was much more pronounced on the *cpm2* roots than the WT roots; *cpm2* had a better overall root architecture and it seems to have the machinery to sustain drought stress better.

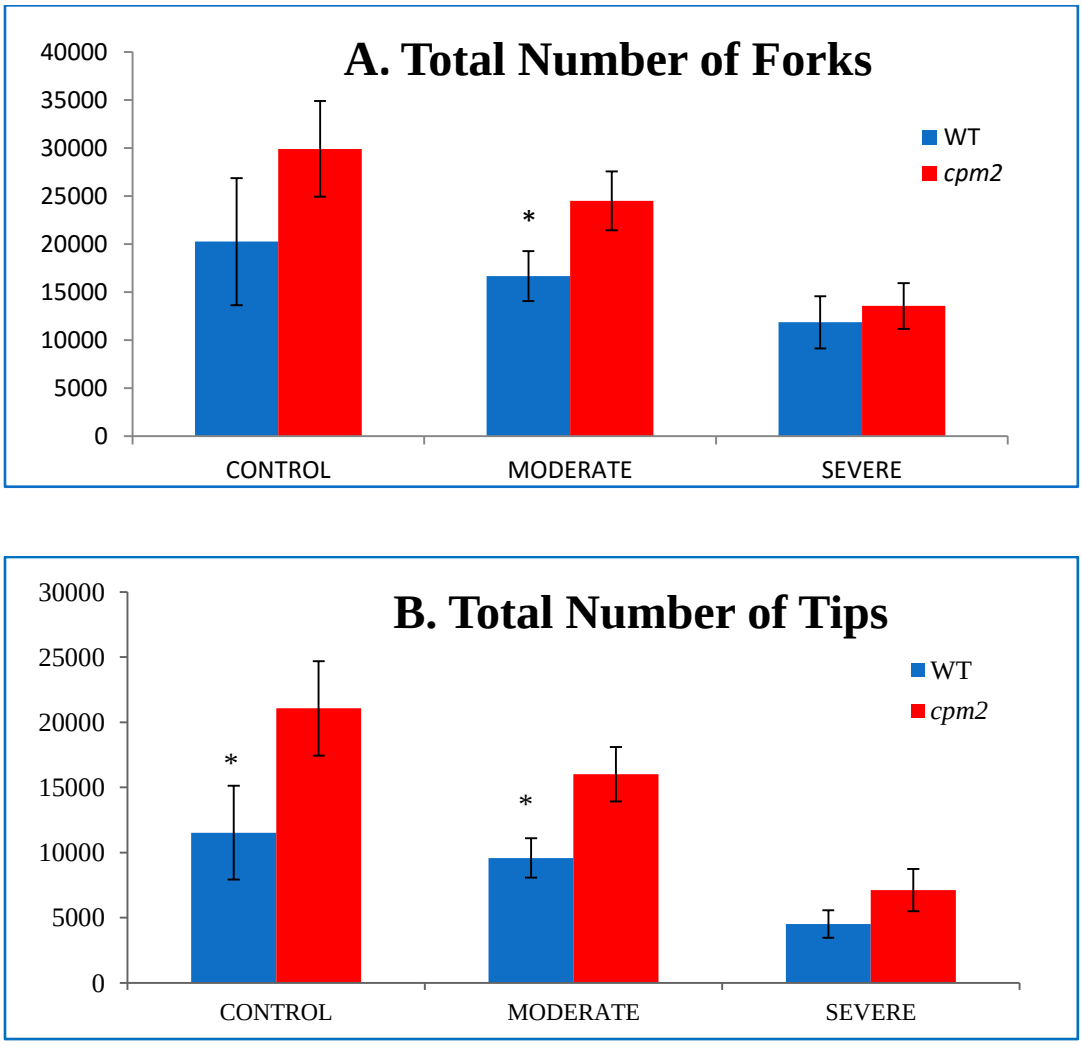
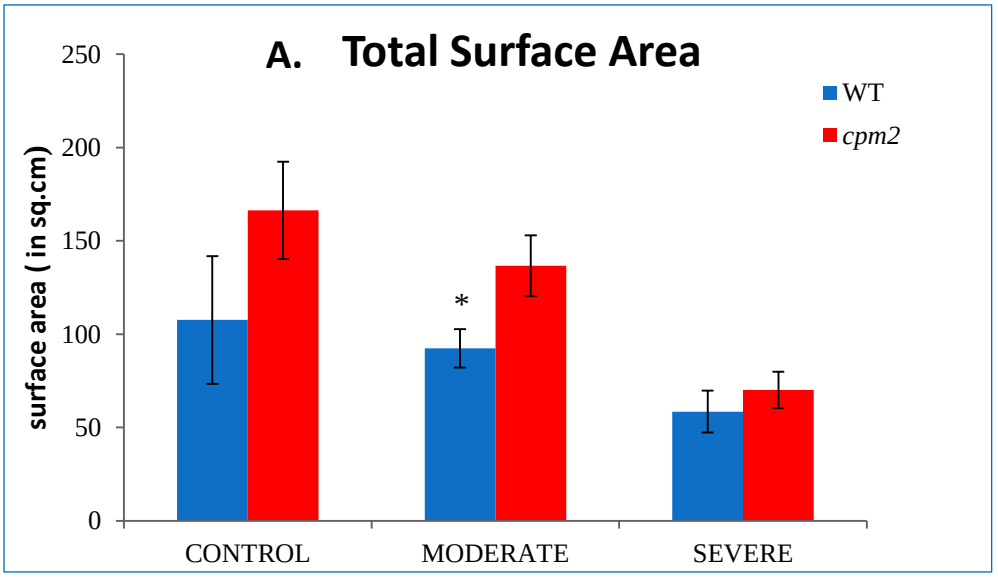



**Figure 12. A) Total Number of Forks and B) Total Number of Tips are shown here.** Plants of two genotypes were grown in soils at different moisture contents**.** Significant differences were observed across the treatments in which *cpm2* seemed to be performing better. Data are mean values ± standard error of five replicates. Stars denote statistical significance ($P \leq 0.05$) between genotypes across treatments in a student's *t*-test.

Total Number of Forks and Total Number of Tips were calculated in both *cpm2* and WT after scanning the roots using the Winrhizo system. It pointed out that *cpm2* in the three different treatments showed much higher total number of forks and tips than the corresponding WT treatments (Figure 12 A & B) and specifically *cpm2* had higher total number of forks and tips under moderate drought as compared to WT under same condition. This might help higher soil exploitation efficiency by deploying finer roots in a root branch system in *cpm2* - which could prove as an advantageous strategy under limiting water conditions.



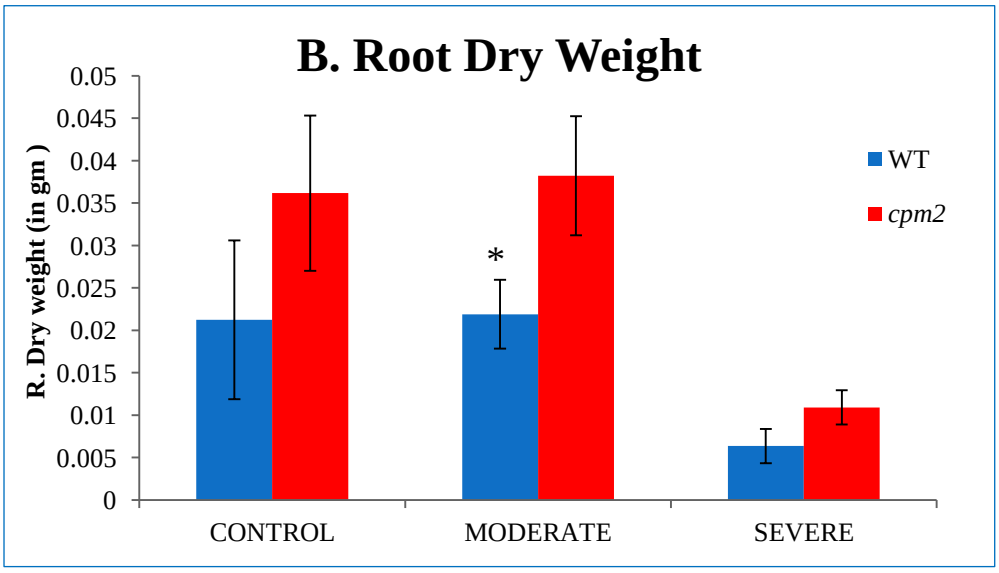




**Figure 13. A) Surface Area B) Root Dry Weight is shown here**. Plants of two genotypes were grown in soils at different moisture contents**.** Significant differences were observed across the treatments in which *cpm2* seemed to be performing better. Data are mean values ± standard error of five replicates. Stars denote statistical significance ($P \leq 0.05$) between genotypes across treatments in a student's *t*-test.

Total Surface Area (TSA) was measured in WT and *cpm2* under control, moderate and severe drought treatments. Based on these measurements, it can be stated that the both WT and *cpm2* plants showed a reduced surface area as the severity of the treatment increased. Under control condition, TSA in *cpm2* was 35% more as compared to WT however under moderate stress *cpm2* had 32% more surface area than the WT under the same treatment ($P \leq 0.05$). Under severe stress, the difference became almost similar but it was greatly reduced in both the genotypes (Figure 13A).

Root Dry Weight (RDW) was also measured in all the treatments. Under control condition, *cpm2* RDW measured 40% more than the WT under the same condition, However effect of moderate stress treatment was more pronounced on WT as cpm2 had 44% more RDW than WT under the same treatment($P \leq 0.05$). Although under severe stress treatment, the effect of stress was more pronounced on both the genotypes and RDW was greatly reduced but still *cpm2* maintained 45% more RDW than WT. (Figure 13B).

In summary, several root architecture measurements indicated that *cpm2* roots are more developed than those of the WT. Also, *cpm2* is able to maintain a better growth and higher degree of branching under moderate drought conditions

### 3.1.3 Water use efficiency, stomatal conductance, and abscisic-acid levels indicate improved drought tolerance in *cpm2*

Phenotypically, *cpm2* seemed to cope better with drought stress (Figure 9). Therefore, we analyzed water use efficiency (WUE) and stomatal conductance of WT and *cpm2* under drought stress (DS) over the time. WUE can be defined as the ratio of water used by the plant for its leaf area development to the total water uptake. Overall, WUE tended to be significantly higher in *cpm2* as compared to WT under drought conditions (Figure 14A). Initially at 36 days after sowing (DAS), at the initiation of drought treatment, *cpm2* and WT showed similar WUE under control and in DS treatment. Following the drought treatment progressively from 41 – 46 DAS, *cpm2* showed better WUE than WT, with the highest at the most severe drought stress treatment (46 DAS). Till the end of the drought stress experiment, *cpm2* plants maintained better WUE than the WT under DS condition. On the other hand in control condition, difference between WUE of *cpm2* and WT was almost non-significant (Figure 14 B). Thus it could be emphasized here the ability of the *cpm2* plants to

maintain better WUE throughout the drought stress treatment – indicating its physiological capacity to sustain drought better.

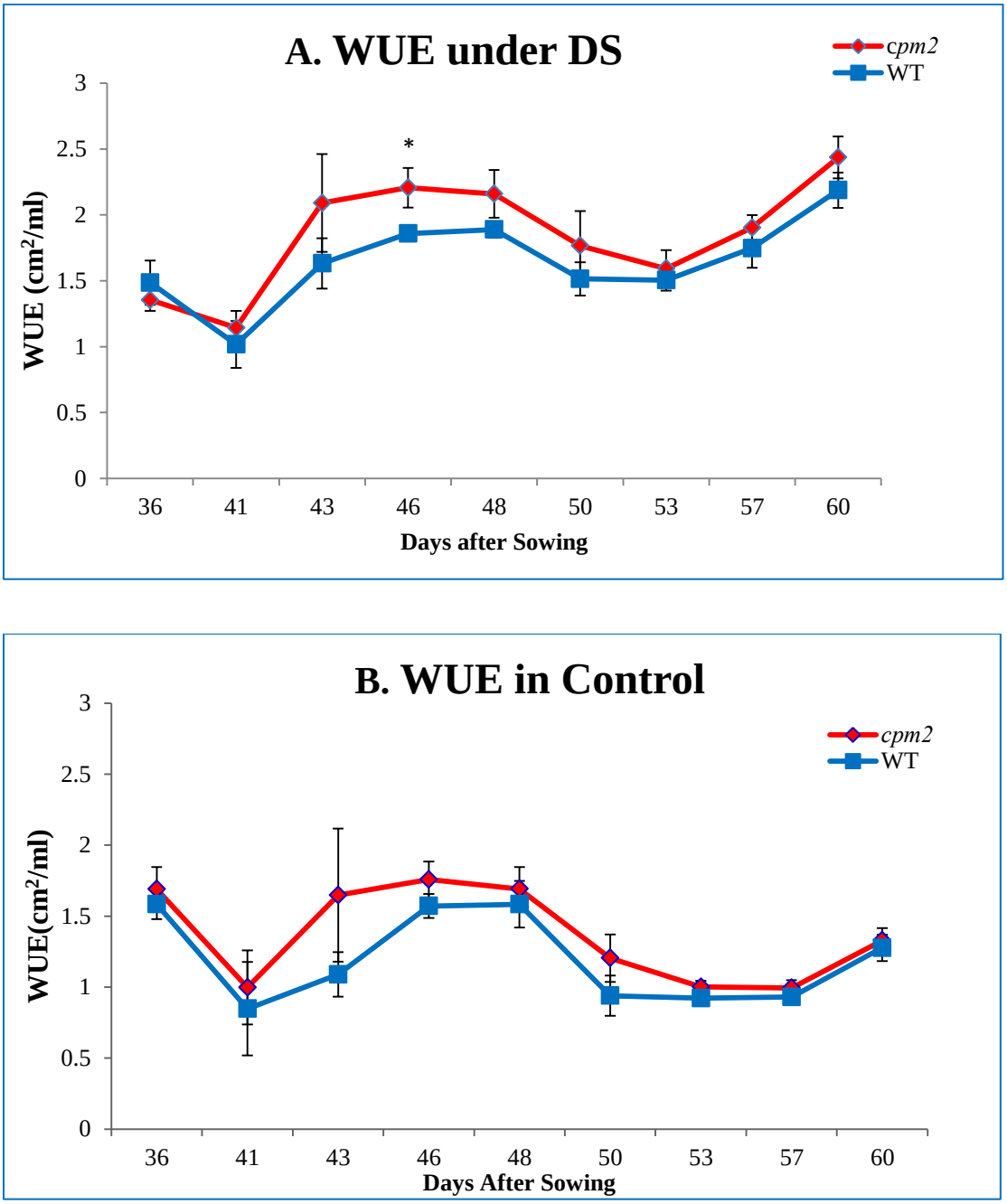
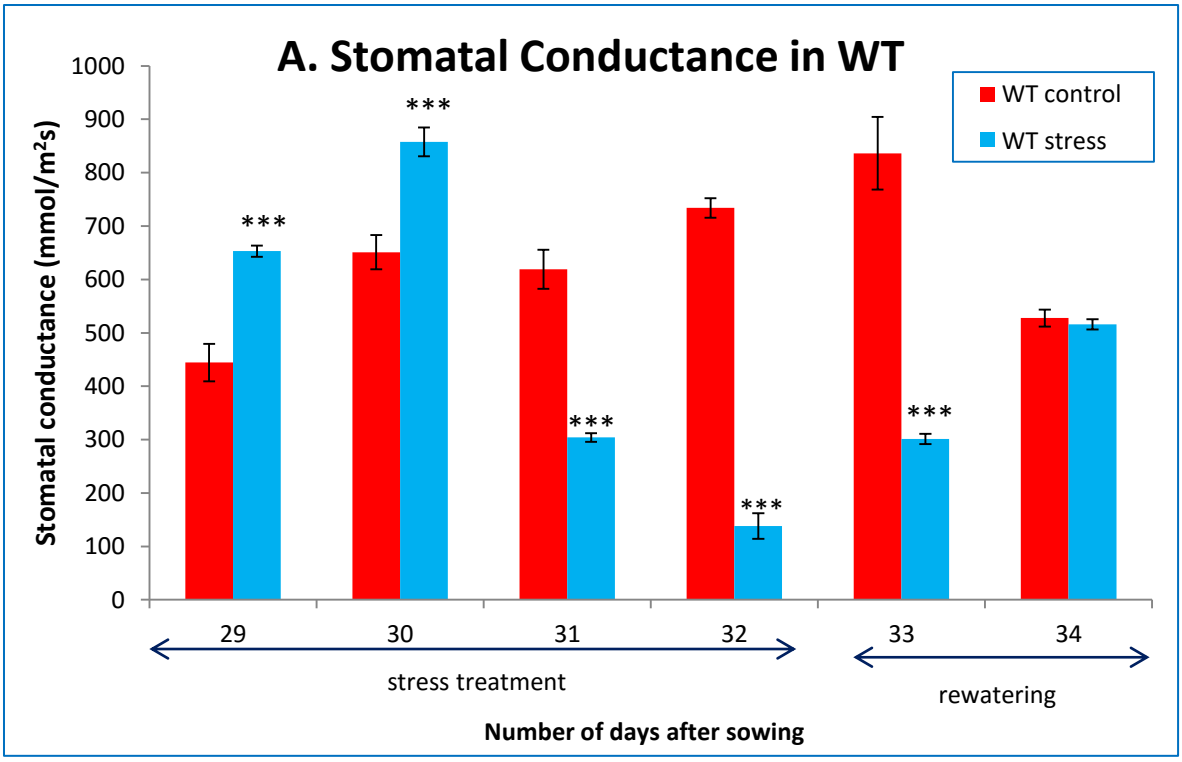



**Figure 14. Water use Efficiency of WT and *cpm2* calculated after normalizing water uptake rates with Leaf Area under A) Drought stress B) Control condition.** WT & *cpm2* plants were drought stressed in lysimeters after 32 Days of Sowing by withholding water. Weighing for lysimeters started from 36 DAS for 3 times a week and controls were replenished upto the target weight after each successive measurement. Plants were photographed at the same time for green leaf area, which was later used to calculate the Water Use Efficiency (WUE). Star (*) denote statistical significance ($P \leq 0.05$) respectively between genotypes in a student's *t*-test.



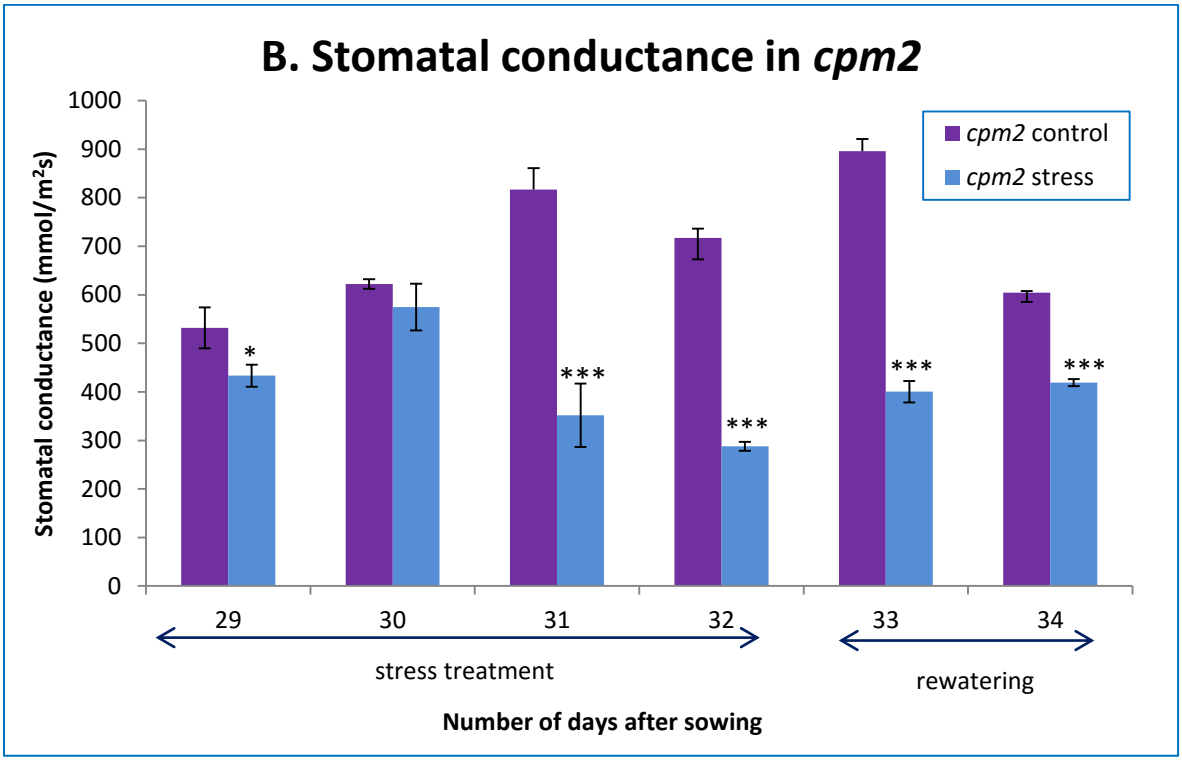




**Figure 15 A) Measurement of Stomatal Conductance in WT B) Measurement of Stomatal Conductance in *cpm2* under control, moderate and severe stress.** Four week old WT and *cpm2* plants were drought stressed in phytochamber by withholding water for four days and then rewatered by replenishing the water levels. Values represent the mean of at least three independent experiments ±SE. Stars (*, ** and ***) denote statistical significance ($P \leq 0.05$, $P \leq 0.01$ & $P \leq 0.001$) respectively between genotypes in a student's *t*-test.

The stomatal conductance measures the rate of passage of gases such as carbon dioxide ($CO_2$) or water vapour through the stomata of a leaf, and is a function of the density, size, and aperture of stomata. Stomatal conductance was measured in WT and *cpm2* under both control and DS treatments, respectively. Measurements were started on 28 days old plants. In control conditions, WT and *cpm2* maintained almost a steady state throughout the course of experiment (Figure 15 A & B).

However, under drought treatment, there was a degree of variation in the stomatal responses of both WT and *cpm2* as observed through the measurements of the stomatal conductance. For instance, WT was having higher stomatal conductance (by 30-40%) as compared to the *cpm2* on 29$^{th}$ day and 30$^{th}$ day (1 day and 2 days after drought treatment respectively). On 31$^{st}$ day (3 days after treatment), stomatal conductance in both WT and *cpm2* dropped significantly but both maintained almost the same degree of conductance. Interestingly, on 32$^{nd}$ day (4 days after treatment), the trend became reverse. Here, *cpm2* showed higher stomatal conductance (3 fold than WT) and WT was almost wilting (also evident from the phenotype, figure 9C). After re-watering, both the plants replenished their water levels but on the 33$^{rd}$ day (1 day after re-watering), *cpm2* still had higher degree of conductance. On 34$^{th}$ day (2 days after rewatering) WT and *cpm2* recovered completely and maintained a stomatal conductance as in the control treatment. However *cpm2* maintained a constant stomatal conductance throughout the re-watering treatment (Figure 15 A & B).

As hormonal response characteristic for drought stress and to understand the drought effect on the stomatal conductance better, ABA levels were quantified. ABA levels in WT and *cpm2* shoot tissue under 3 different conditions of control, moderate stress (SMC 30%) and severe stress (SMC 20%) were measured. Under control and moderate stress conditions, WT accumulated almost the double amount of ABA as compared to *cpm2*. However, severe stress (20% SMC) led to an increase of ABA in both, *cpm2* and WT. While the content in the WT increased approximately 4-fold, it increased by a factor of almost 16 in *cpm2* shoots. Under these conditions, *cpm2* contained approximately 2-fold the amount of ABA compared to the WT (Figure 16).

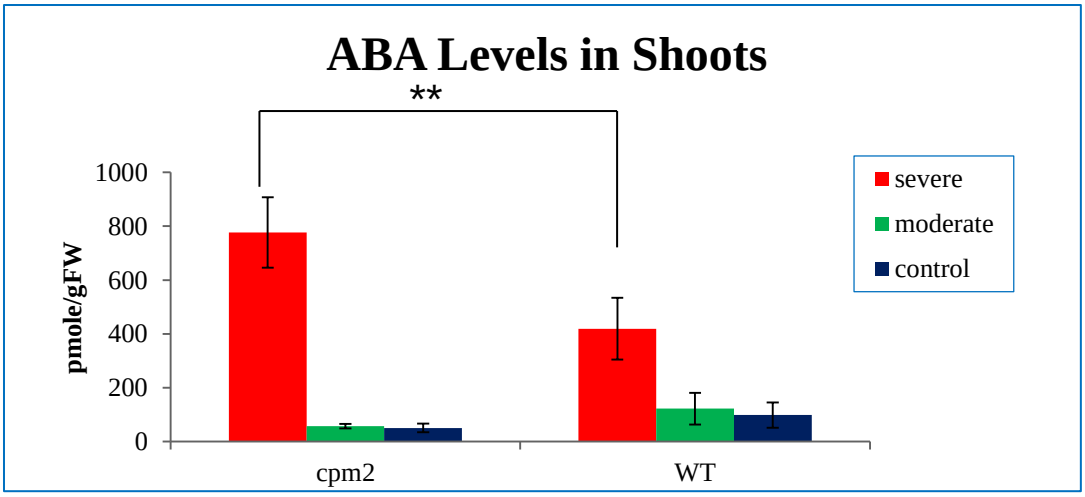



**Figure 16. Measurement of shoot ABA levels in *cpm2* and WT under control, moderate and severe drought stress.** Values represent the mean of at least three independent experiments ±SE. Stars (**) denote statistical significance ($P \leq 0.01$ ) respectively between genotypes in a student's *t*-test.

From the measurement of ABA levels in shoots, it could be emphasized that with the increase in severity of drought stress, ABA levels exaggerated in *cpm2* and quite interestingly, they became nearly 2-folds in *cpm2* under severe stress as compared to WT under severe stress. In contrast, under moderate stress, the mutant accumulated much less ABA than the wild type, indicative of a lower level of drought-stress induced imbalance.

### 3.2. Description of the proteome profiles of mutant versus wild type as identified by TMT proteomics.

#### 3.2.1 Assessing the function of jasmonate for the drought response in rice roots by proteome approach

As roots are the primary organ to perceive drought stress in the plant, a detailed morpho-physiological analysis of roots was carried out as mentioned above. However in order to gain better insights into understanding of the key molecular players underlying such potential differences between WT and *cpm2,* a proteomic approach was envisioned. We therefore decided to perform a comparison of the root proteomes of both genotypes. Proteins of the rice roots from both the genotypes that were subjected to severe drought stress along with its respective control well water treatments, were collected and used for high throughput Tandem Mass Tag (TMT) analysis subsequently.

### 3.2.2 Proteome analysis by TMT reveals unique and common drought regulated proteins

A total of 71,331 spectra were detected by TMT and 15,172 were identified. Among the identified spectra, 13,485 matched 4,573 peptides, with 4,194 hits assigned to unique peptides. In total, 1578 proteins were identified by MS/MS in both WT and *cpm2* roots together. Out of these, 351 proteins were uniquely found in WT roots, and 341 proteins were unique to *cpm2* roots. 443 proteins were the common proteins which were reported in the root tissue of both the genotypes (Figure 17 A). However, the number of proteins which were quantitatively analyzed during MS-MS were 272 and 217 in WT and *cpm2* respectively, and 319 proteins were common proteins at the quantitative scale (Figure 17 B) The proteins from rice roots with or without drought stress treatment were then subsequently analyzed using Mapman ontologies. For this purpose, a threshold of log ratio of 1.0 or greater (10-fold) was used as criterion for a protein being considered as more abundant. On the other hand, proteins having log ratios less than 1.0 (reduction by at least a factor of 10) were considered as less abundant.

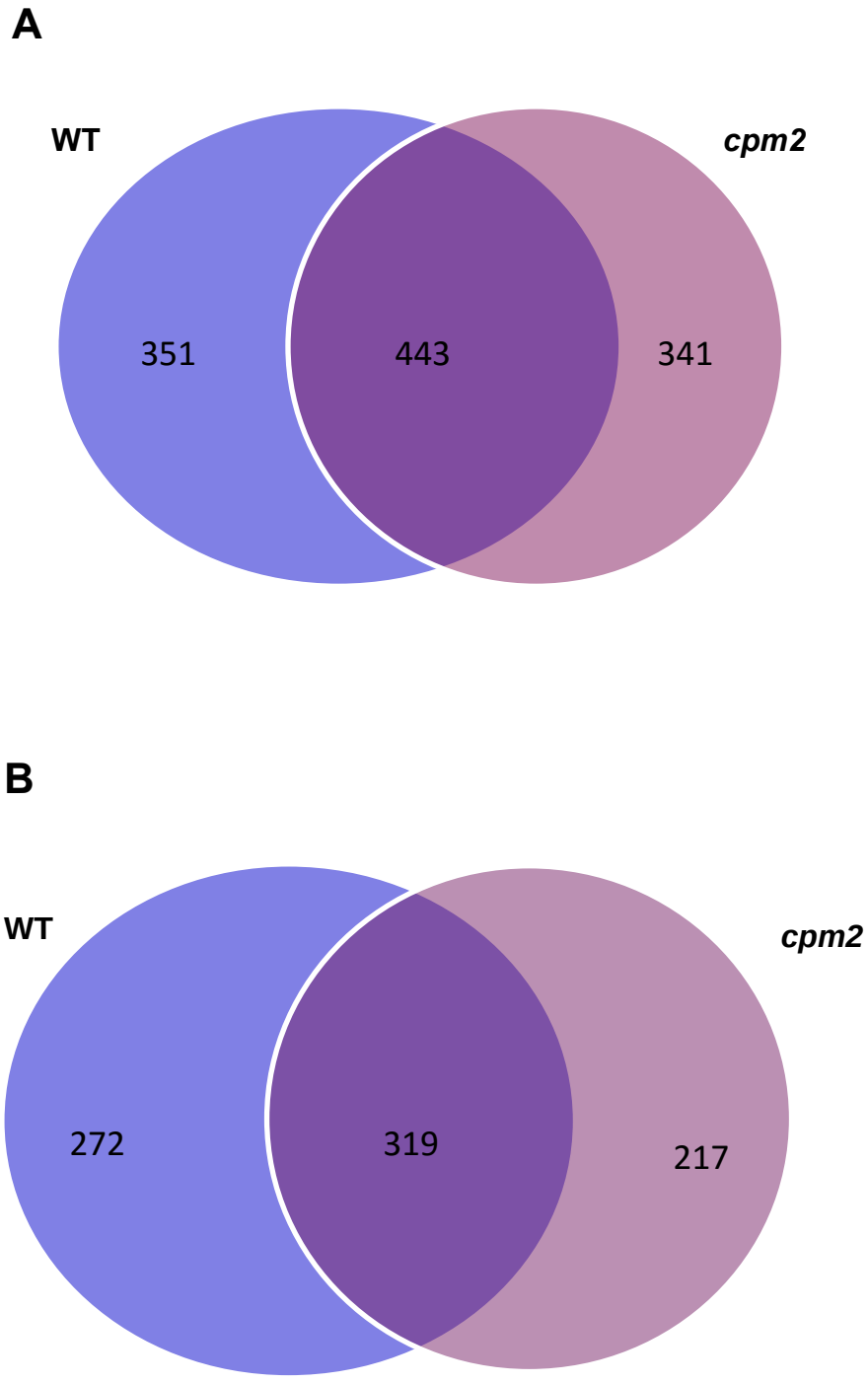



**Figure 17 A)** Venn diagram depicting the unique and common proteins identified in WT and *cpm2* roots during TMT analysis **B)** Venn diagram depicting the number of proteins quantified during TMT analysis

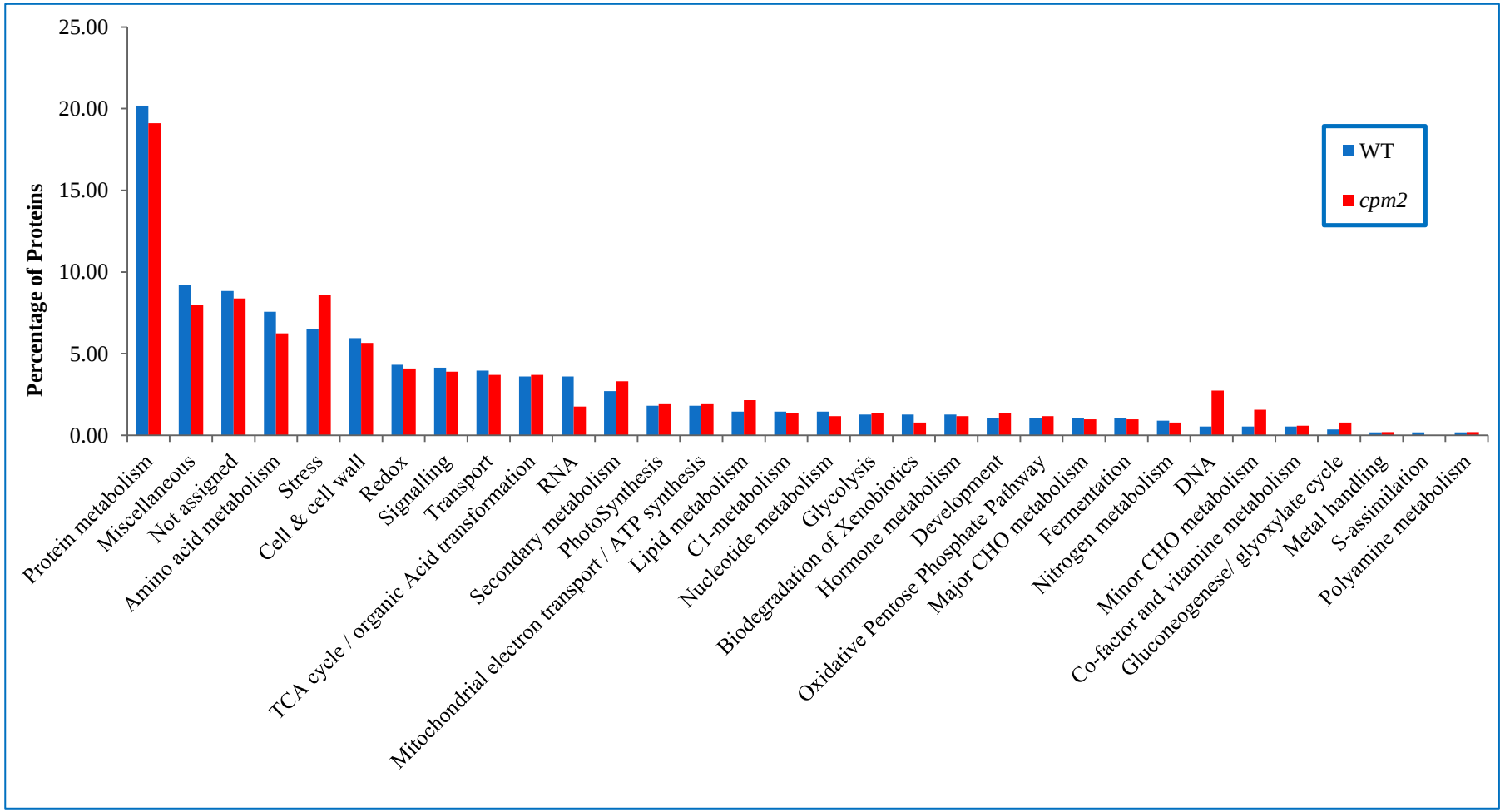



**Figure 18. Overview of the percentage of root proteins mapped onto gene ontologies through MAPMAN in WT and *cpm2*.** Proteins identified after TMT analysis were classified into 32 functional categories according to MAPMAN ontology

### 3.3. MapMan based ontological classification of proteins identified in WT and cpm2 roots

A dynamic range of proteins was identified as implicated by the coverage of proteins from a wide range of isoelectric points (4.41–11.91) and molecular weights (4.4 – 236.7 kDa). In order to understand the functional importance, these proteins were further classified into different gene ontology (BINs) using an *in silico* tool. MAPMAN tool was used to assign these proteins from both the genotypes into 32 functional categories according to rice mapping file (Thimm *et al.* 2004). The overview of percentage of proteins falling under these functional categories in *cpm2* and WT are shown in the figure 18. Uniquely represented proteins in WT and *cpm2* are listed in supplementary Table 1 and 2, respectively.

Among a subset of differentially expressed proteins, few were assigned to a couple of important ontologies, namely; energy metabolism and protein metabolism. We also observed some interesting proteins assigned in the miscellaneous category of the MAPMAN too.

#### 3.3.1 Energy Related Proteins:

Energy related proteins were more abundant in *cpm2* and were less abundant in WT. Triosephosphate isomerase and glyceraldehyde-3- phosphate, major proteins involved in glycolysis, were more abundant in *cpm2* than the WT. Similarly, pyruvate decarboxylase, an important protein involved in fermentation process was more abundant in *cpm2* as compared to WT (figure 19)

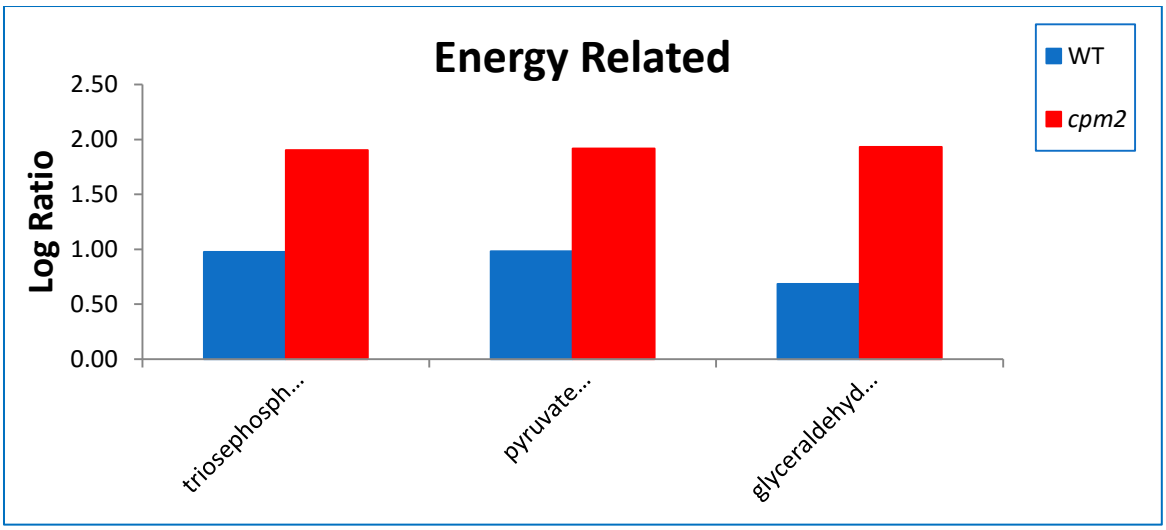



**Figure 19. Differentially expressed proteins related to Energy.** Differential proteins related to energy were abundant in *cpm2* as evident from the log ratios (stress/control) obtained from the TMT analysis.

### 3.3.2 Proteins involved in protein metabolism:

Two peptidase T1 family proteins were also more abundant and differentially expressed in cpm2. Also, a 60S ribosomal protein was also more abundant and differentially expressed in cpm2 (Figure 20).

### 3.3.3 Proteins from the miscellaneous category:

A small number of proteins which were not assigned under any major functional category were classified as miscellaneous. An aquaporin known as OsPIP1-2(LOC_Os04g47220) was more abundant in WT as compared with *cpm2* (Figure 21). Conversely, a Ras-related protein, OsRas1(LOC_Os01g37800 belonging to family of small GTPase was more abundant in *cpm2* as compared to WT (Figure 21) A protein named 3-isopropylmalate dehydratase small subunit 2(LOC_Os02g43830) , which is known to be involved in biosynthesis of leucine, was also found to be more abundant in *cpm2* than the WT (Figure 21). Similarly, OsGS1 (LOC_Os03g12290), a key protein involved in N-metabolism in plants was also highly abundant in *cpm2* as compared to WT (Figure 21).

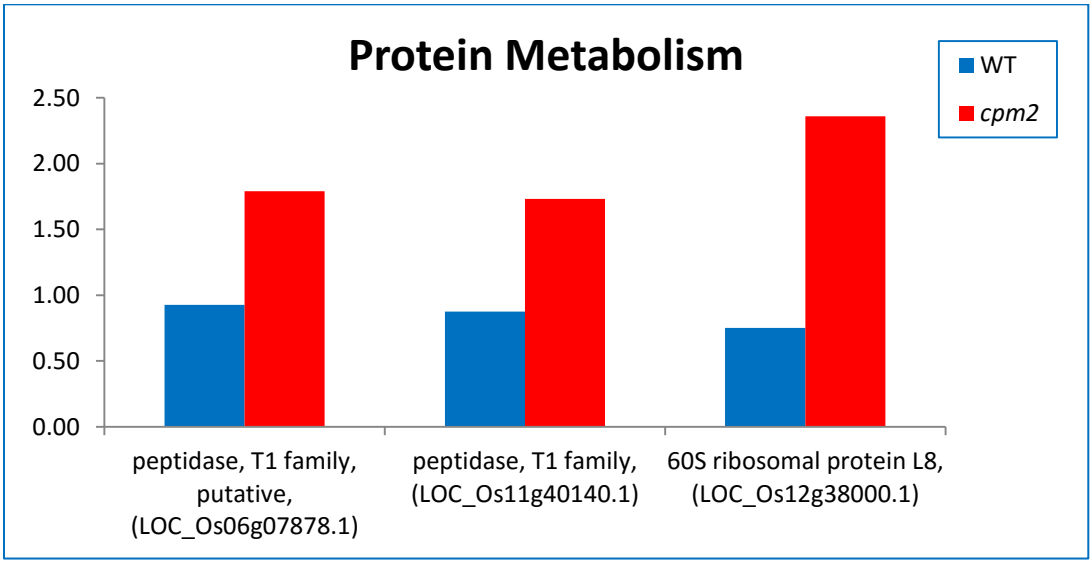



**Figure 20. Differentially expressed proteins related to protein metabolism.** Differential proteins related to protein metabolism were abundant in *cpm2* as evident from the log ratios (stress/control) obtained from the TMT analysis.

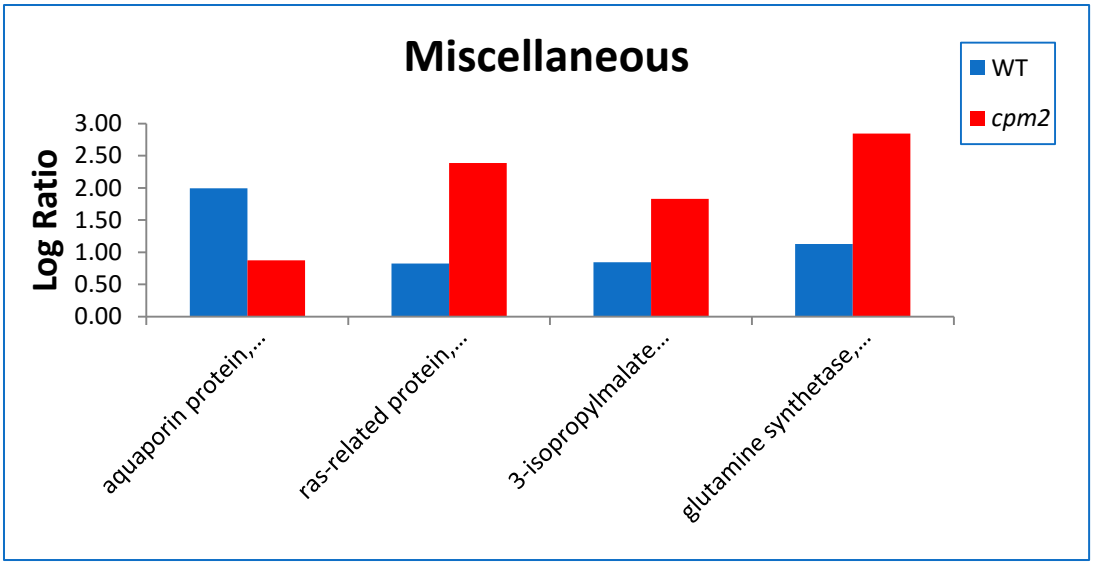



**Figure 21. Differentially Expressed Proteins belonging to the miscellaneous category**. Aquaporin was only abundant in WT whereas rests were abundant in *cpm2* as evident from the log ratios (stress/control) obtained from the TMT analysis.

On the basis above mentioned differentially expressed proteins- it can be suggested that cpm2 has better metabolism. This can be useful for *cpm2* to maintain its homeostasis under stress.

### 3.4. Cross-connection of these clusters according to functional categories

#### 3.4 1. Understanding of key enzymes invloved in JA synthesis in response to drought stress

#### 3.4.1.1 Abundance of JA biosynthesis enzymes was differentially affected in WT and *cpm2*

TMT technique employed to analyze the whole root proteome also shed light on jasmonate-specific proteomic response to drought stress in WT and *cpm2*. While looking for proteins specifically involved into the JA biosynthesis pathway, it was a surprise to observe that the AOC protein, a key enzyme involved in JA biosynthesis pathway became highly abundant in WT (Figure 22A) but it was not detected in *cpm2* at all. The qRT-PCR was also performed to determine whether the observed changes in protein abundance were regulated at the transcriptional level. Transcript abundances of mRNAs encoding AOC protein were analyzed at control, moderate and severe drought stressed conditions. Under control condition, level of the *AOC* mRNA transcript in WT was more than *cpm2* (nearly 2 folds) but the trend got reversed when *cpm2* transcripts became more up-regulated than WT under moderate and severe conditions (Figure 22B). However, it is important to mention here that the transcripts

accumulating in *cpm2* do not give rise to a functional AOC as *cpm2* mutant is generated by deleting 11 base pairs in the first exon of the AOC gene, leading to the loss of 3 amino acids and a subsequent frameshift mutation (Riemann *et al.,*2013).In contrast to AOC, OPR7 protein, a key enzyme operating downstream of AOC, became more abundant as compared to WT under drought condition (Figure 23A). This is surprising as in *cpm2* the natural substrate for OPR7, 12-oxo-phytodioneic acid (OPDA), is not present due to the absence of AOC protein (Riemann *et al.,*2013). The levels of mRNAs encoding *OPR7* were also checked at control, moderate and severe drought stress condition. Similar to *AOC* transcript, the transcript levels for *OPR7* were lower in *cpm2* in control treatment as compared to WT. However, under moderate and severe stress, *OPR7* transcripts in *cpm2* were slightly up-regulated (but non-significant) but remained almost similar to mRNAs levels of *OPR7* in WT. (Figure 23B). Hence, it means that the upregulation of the protein occurs at the post-transcriptional level.

Overall summing up together, it was observed that the protein for AOC, an enzyme upstream in the JA pathway, which was highly abundant in WT, but it was not reported at all in *cpm2* during TMT analysis. For OPR7 which is the next successive enzyme in the pathway, the protein was found to be more abundant in *cpm2*. The transcript abundances for both the above mentioned proteins were also determined successfully using the qRT-PCR.

Therefore the dynamics of the enzymes AOC and OPR7, which are present up-stream and down- stream of OPDA respectively, seem to be affected by drought stress. This could be very crucial – considering the recently studied role of OPDA as a signaling molecule for plant response against environmental cues (Savchenko *et al*.,2014).

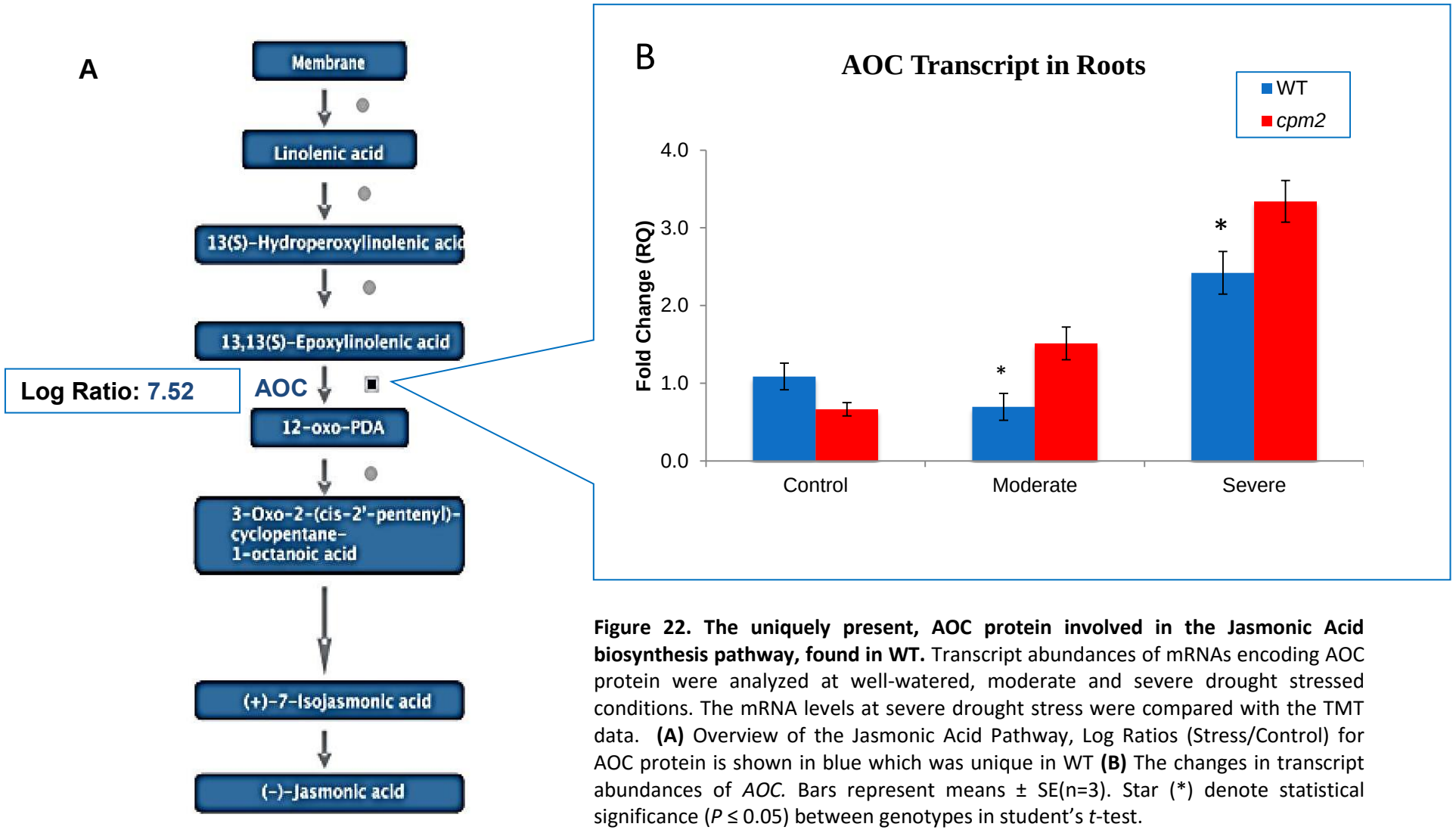
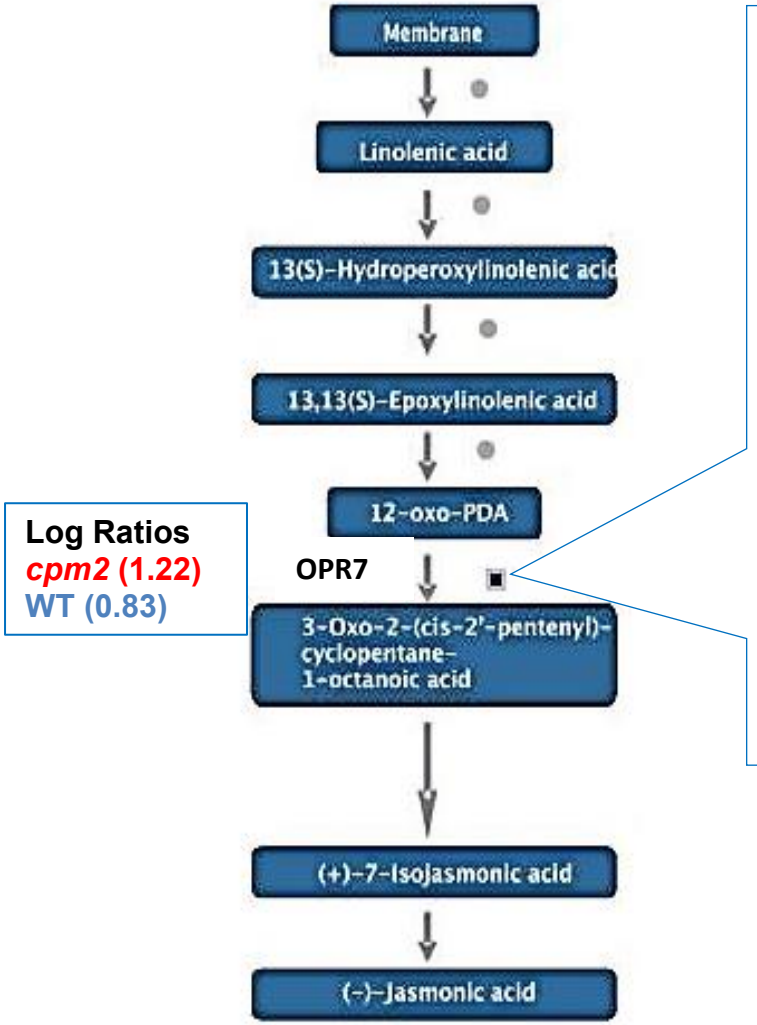



**Figure 22. The uniquely present, AOC protein involved in the Jasmonic Acid biosynthesis pathway, found in WT.** Transcript abundances of mRNAs encoding AOC protein were analyzed at well-watered, moderate and severe drought stressed conditions. The mRNA levels at severe drought stress were compared with the TMT data. **(A)** Overview of the Jasmonic Acid Pathway, Log Ratios (Stress/Control) for AOC protein is shown in blue which was unique in WT **(B)** The changes in transcript abundances of *AOC.* Bars represent means ± SE(n=3). Star (*) denote statistical significance ($P \leq 0.05$) between genotypes in student's *t*-test.



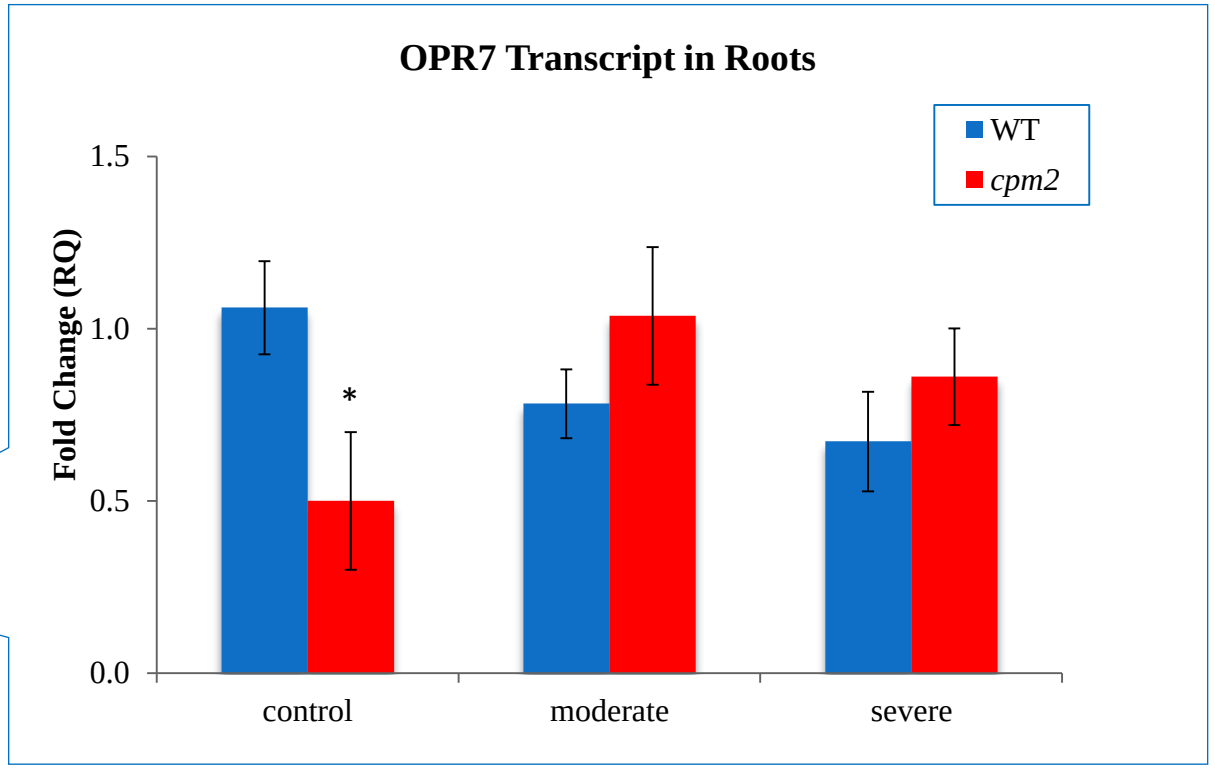




**Figure 23. The differentially expressed OPR7 protein involved in the Jasmonic Acid biosynthesis pathway.** Transcript abundances of mRNAs encoding OPR7 protein were analyzed at well-watered, mild and severe drought stress conditions. The mRNA levels at moderate and severe stress were compared with the TMT data. **(A)** Overview of the Jasmonic Acid Pathway, Log Ratios ( Stress/Control) for OPR7 protein are shown in red and blue respectively for cpm2 and WT **(B)** The changes in transcript abundances of *OPR7.* Bars represent means ± SE (n=3). Star (*) denote statistical significance ($P \leq 0.05$) between genotypes in a student's *t*-test.

### 3.4.2: ROS detoxification proteins were found to be more abundant in the mutant

Apart from looking at unique proteins that were regulated in these two genotypes in response to drought, we also explored the common proteins that were differentially accumulated between the two genotypes. In order to identify those proteins, four different comparison studies were employed. Namely,

1. WT down-regulated vs *cpm2* up-regulated
2. WT up-regulated vs *cpm2* down-regulated
3. *cpm2* up-regulated vs WT up-regulated
4. *cpm2* down-regulated vs WT down-regulated

Proteins showing a difference of log ratio of 1.0 or greater (~ 10 fold-change) were identified and further examined to determine their role in response to drought stress. Interestingly many of these proteins belong to redox machinery. This ontology contains six proteins, five of which were more abundant in *cpm2* under drought stress and only one was found to be more abundant in WT. Abundant proteins included glutathione S-transferases (OsGSTF2 and OsGSTU12), ascorbate peroxidase (OsAPX7), a DJ-1 family protein and serine hydroxymethyltransferase (SHMT). The only abundant protein in WT under this category was the non-symbiotic haemoglobin 2 (nsHb2) (Figure 24).

Glutathione-S-transferases catalyze conjugation of tri-peptide glutathione (GSH) to a variety of hydrophobic, electrophilic and cytotoxic substances and thus they help to inactivate cytotoxic compounds in plants. *OsGSTF2* also known as GST-II, and *OsGSTU12* became more abundant in *cpm2* under stress as compared to WT (Figure 24).

Ascorbate peroxidases (APX) are evidently known to be the enzymes that detoxify peroxides such as hydrogen peroxide using ascorbate as substrate. One of this group of proteins *OsAPX7* became more abundant in *cpm2* as compared to WT (Figure 24).

DJ-1 family proteins are reported to have an anti-oxidant property and their loss of function have been shown to result in an accelerated cell-death. One of these DJ-1 family proteins was also more abundant in *cpm2* as compared to WT (Figure 24).

Serine hydroxymethyltransferases (SHMTs) are also known to be part of dissipatory mechanisms to minimize production of reactive oxygen species (ROS) in plants. Protein for one such SHMT, *OsSHMT2* was more abundant in *cpm2* as compared to WT (Figure 24).

Non-symbiotic hemoglobin2 was the only protein less abundant in *cpm2* falling under redox category (Figure 24). Limited information is available about this class of protein and also its probable function under abiotic stress but it is known to be occurring under hypoxic conditions in plant roots.

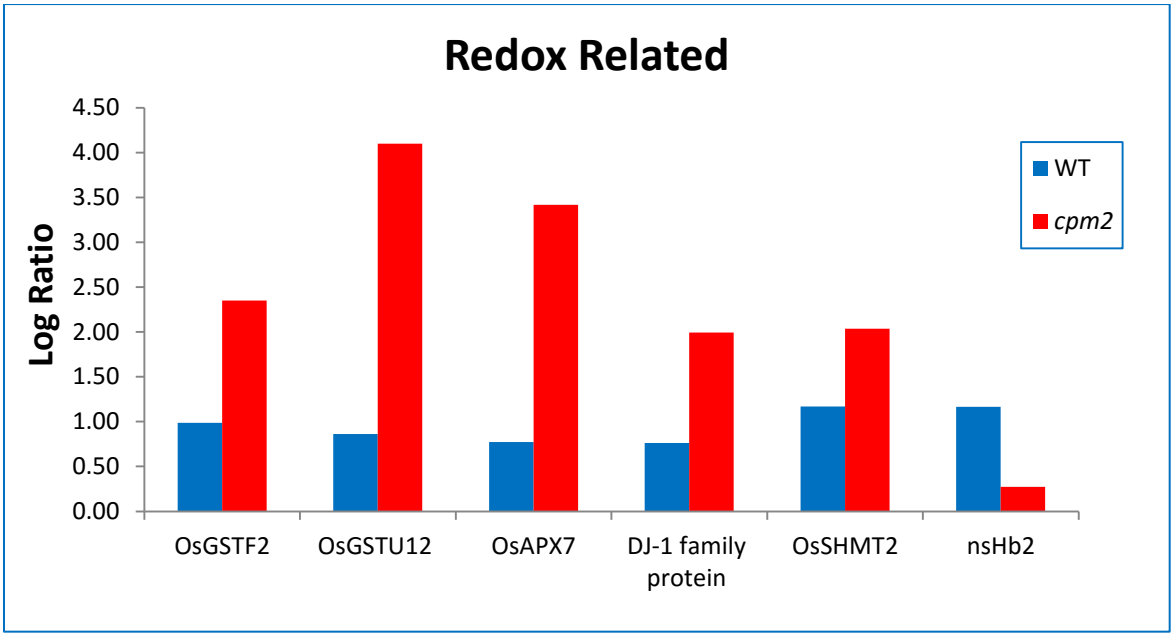



**Figure 24. Differentially Expressed Proteins belonging to the redox machinery.** Most of the proteins related to redox machinery were abundant in *cpm2* as evident from the log ratios (stress/control) obtained from the TMT analysis.

### 3.4.3 Under drought stress, *cpm2* roots accumulate more cell organization and cell wall related proteins

Overall, three differentially expressed proteins were mapped onto cell wall related category. A protein belonging to the glycosyl hydrolases family 17 known as Gns6 was more abundant in *cpm2* than the WT (Figure 25). Also, the O-methyltransferase (ROMT-9) was more abundant in cpm2 as well. Similarly, another cell wall biosynthesis related protein; UDP-glucose-6-dehydrogenase was highly abundant in *cpm2* than the WT (Figure 25).

Two differentially expressed proteins were categorized as cell organization proteins because of their role in cell organization. β-Tubulin 1, a major building block of microtubules was more abundant in *cpm2* as compared to WT. Similarly, the actin nucleation protein was more abundant in *cpm2* as compared to the WT (Figure 26)

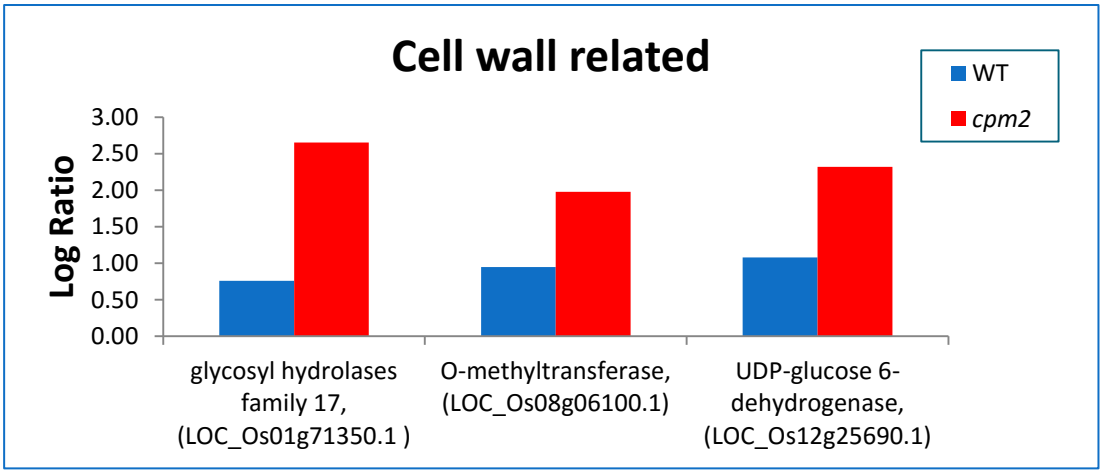



**Figure 25. Differentially Expressed Proteins belonging to the cell wall metabolism**. Differentially regulated proteins related to cell wall were abundant in *cpm2* as evident from the log ratios (stress/control) obtained from the TMT analysis.

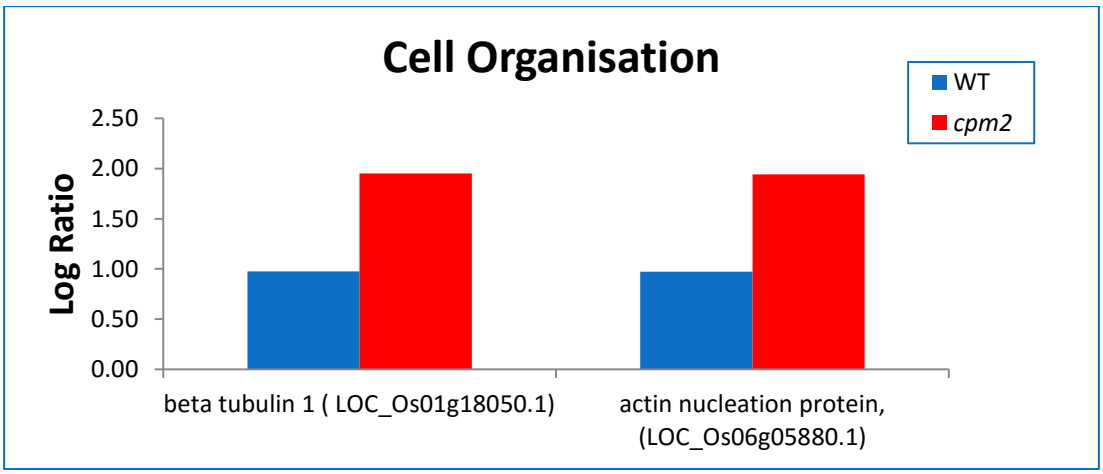



**Figure 26. Differentially expressed Proteins belonging to the cell organization category**. Differential proteins related to cell organization were abundant in *cpm2* as evident from the log ratios (stress/control) obtained from the TMT analysis.

### 3.4.3.1 Proteins participating in Phenylpropanoid pathway were specifically induced in *cpm2*

As described in the previous section, proteins related to cell organization and cell wall were more abundant in *cpm2*. Moreover, it was also found that the proteins responsible for mediating phenylpropanoid pathway were also more abundant in *cpm2* as compared to WT under drought stress. Interestingly for the enzymes involved at the beginning of the phenylpropanoid pathway, phenylalanine-ammonia-lyase (PAL) was found to be more abundant in *cpm2* under drought stress (Figure 27). Similarly another important protein further downstream in the phenylpropanoid pathway namely, 4-coumarate-CoA ligase

(Os4CL) was more abundant in *cpm2* during stress as compared to control. Interestingly these proteins were not detected in WT and seem to be an unique response of *cpm2* (Figure 27). Likewise proteins specific for the lignin biosynthesis branch of the phenylpropanoid pathway, the first enzyme caffein-CoA-methyltransferase also known as OsOMT26 was only induced in *cpm2* under drought stress. Also cinnamyl alcohol dehydrogenase (OsCAD4) was more abundant in *cpm2* than in the WT under drought stress as well. Lastly, caffein-o-methyltransferase (OsOMT26) was also only induced in WT (Figure 27).

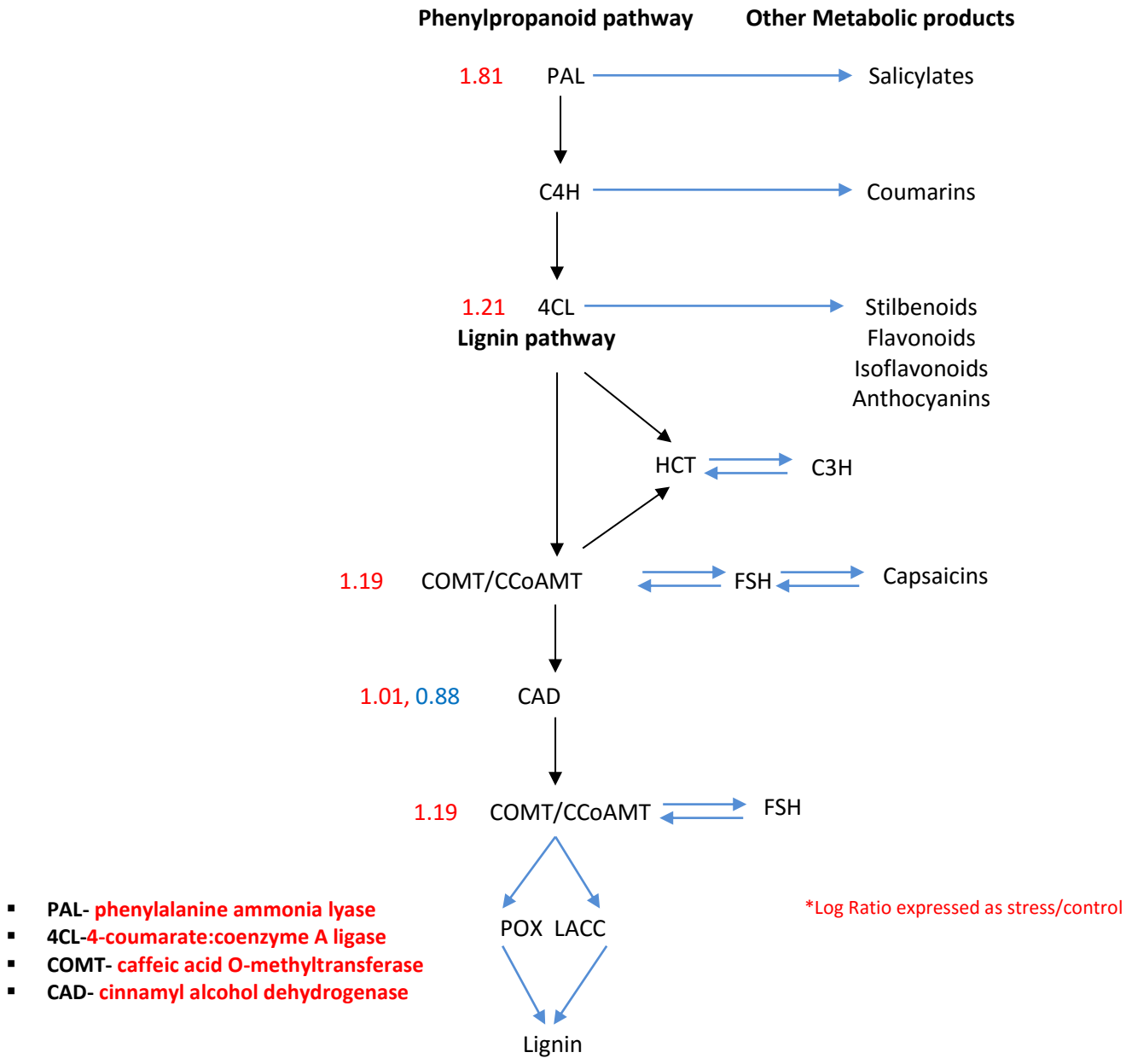



**Figure 27. Simplified diagram of enzymatic steps in pathways committed to Phenylpropanoid pathway with main focus on lignin biosynthesis step:** Log ratios for proteins identified in *cpm2* and WT through TMT analysis are shown in red and blue respectively beside the protein names.

Gene expression of the key genes involved in phenylpropanoid pathway was measured using qRT-PCR analysis. Transcripts encoding for *PAL*, *4CL* and *COMT* were analyzed at control, moderate and severe stress in both the genotypes because the respective proteins had been more abundant.

In case of *OsPAL,* the transcript abundances of mRNAs encoding PAL protein were significantly lower in *cpm2* as compared to WT under control conditions. Similarly, under moderate stress transcript abundances were lower in *cpm2* as compared to WT. But under severe stress, there was no significant difference between WT and *cpm2* (Figure 28). If we compare the relative change in transcript abundances between moderate stress to control, it is quite evident that it correlates with the OsPAL protein abundance in *cpm2* under drought stress however in case of the WT, there was no co-relation in transcript abundances.

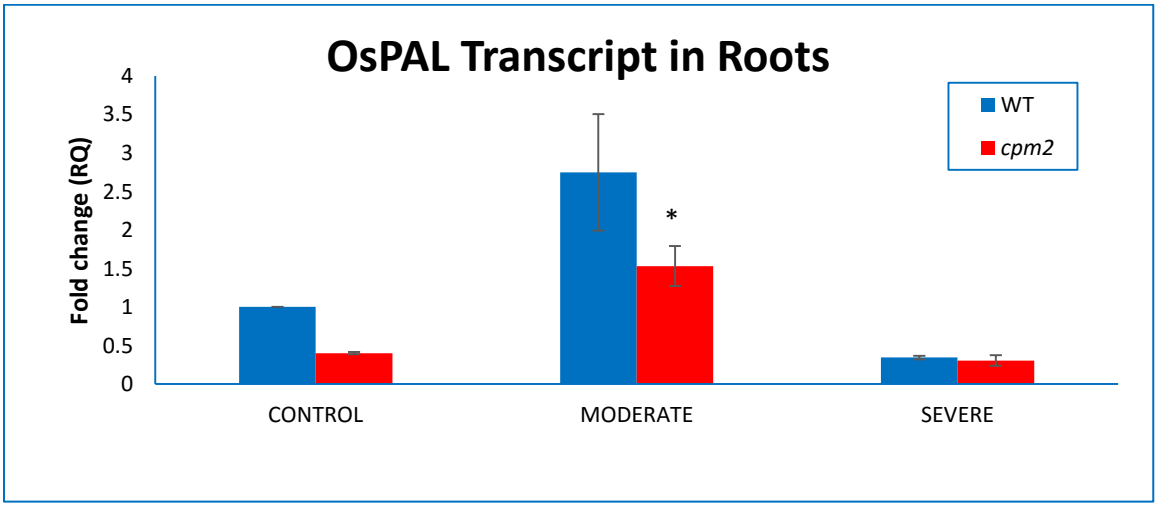



**Figure 28.** Changes in transcript abundance of OsPAL (LOC_Os02g41630) in WT and *cpm2* under control, moderate and severe drought stress conditions. Stars (*) denote statistical significance ($P \leq 0.05$) between genotypes respectively in a student's t-test.

The level of mRNAs encoding 4CL, a gene further downstream in phenylpropanoid pathway, were also checked. As seen in figure 29, transcript levels were comparatively lower in *cpm2* than WT under control condition but interestingly under moderate stress, mRNAs levels increased in *cpm2* significantly as compared to WT. Under severe stress, transcript levels were bit higher in *cpm2* as compared to WT but if compared to moderate stress transcript levels in *cpm2* decreased significantly (Figure 29). By comparing the relative change in transcript abundances between moderate stress to control, it becomes well evident that

relative change (moderate stress/control) correlates with the abundance of 4CL protein in *cpm2* (Figure 27).

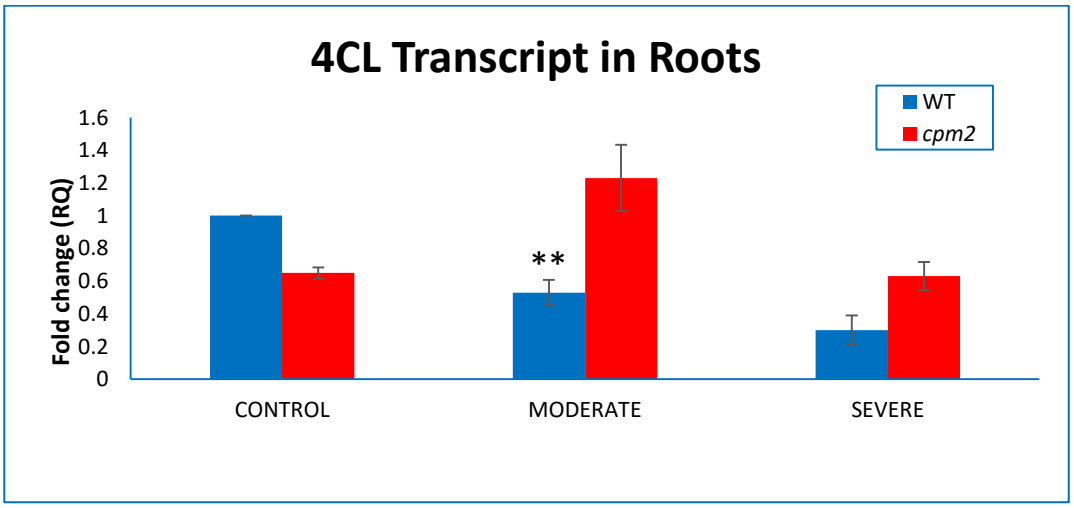



**Figure 29.** Changes in transcript abundance of 4CL (LOC_Os02g08100) in WT and *cpm2* under control, moderate and severe drought stress conditions. Stars (**) denote statistical significance ($P \leq 0.01$) between genotypes respectively in a student's t-test.

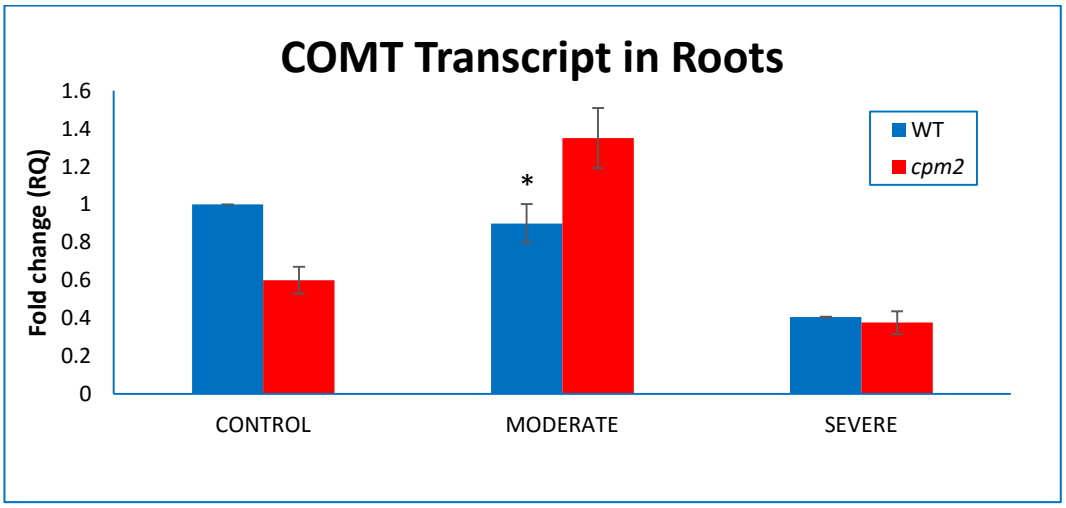



**Figure 30.** Changes in transcript abundance of COMT (LOC_08g38900) in WT and *cpm2* under control, moderate and severe drought stress conditions. Star (*) denote statistical significance ($P \leq 0.05$) between genotypes respectively in a student's *t*-test.

The expression of the gene coding for the protein, COMT was also analyzed which is further downstream of 4CL. It was found that the transcript levels in *cpm2* were comparatively lower under control condition as compared to WT. Under moderate stress, transcript levels in *cpm2* were comparatively higher than levels in *cpm2* under control (2 fold) and hence it

correlates with the proteomic abundance but under severe stress there was no difference between transcript levels in both the genotypes. (Figure 30).

**3.5 Summary of the results:**

In this study, the data generated was intended for comparing the relative changes that are involved in drought-stress adaptation mechanisms of JA deficient mutant, *cpm2* to the WT background rice cultivar Nihonmasari. The data obtained in this study showed the following observations:

1. Morphological investigation revealed that the damage symptoms triggered by drought stress such as leaf rolling and wilting were less pronounced in *cpm2* and hence it showed less sensitivity to drought stress.
2. Significant differences were observed in root architecture across the treatments in both WT and *cpm2* where *cpm2* had higher root dry weight, total surface area, longer nodal and lateral roots and also had better root architecture than the wild type and could maintain this feature even under drought stress.
3. *cpm2* plants were able to maintain better WUE throughout the drought stress treatment and had better water conservation strategy under drought.
4. *cpm2* maintained lower stomatal conductance under drought stress as compared to WT – indicating increased stomatal activity to control water loss. c*pm2* also had comparatively higher ABA levels in the shoots as compared to WT.
5. A TMT approach was also undertaken to determine if the roots of *cpm2* and WT respond differentially to drought at the proteome level.
    a. In JA biosynthesis pathway, it was observed that the protein for AOC, an enzyme upstream in the JA pathway became highly abundant in WT however it was not reported at all in cpm2. This reconfirmed the lack of AOC in the *cpm2*.
    b. A large number of differentially expressed proteins revealed better ROS detoxification and increased cell and cell wall growth in *cpm2* roots as they were abundant in *cpm2* roots.

# 4. Discussion

Dehydration tolerance in plants has been researched using three main approaches:
1) Investigating tolerant systems such as seeds and resurrection plants 2) By studying the effects of stress on agriculturally important plants and 3) By analyzing mutants from model species. The intension of the current work is to compare the JA biosynthesis mutant to the WT under drought stress at the seedling stage, on the level of morphology, physiology, molecular biology and proteomics in order to shed more light on the role of JA during adaptation to drought stress. The observed changes in the morpho-physiological parameters and in the root proteome after exposure to drought, and their prospective cross-connections in better drought adaptation of *cpm2* are discussed in the following subsections, namely:

(i) Phenotypic and morphological characterization provided cues for better drought adaptation in *cpm2*
(ii) Better physiological modulation in *cpm2* correlate with improved drought tolerance
(iii) Functional context of the protein candidates revealed by the TMT analysis.
(iv) Proposed models for mechanisms of drought adaptation in the mutant.

## 4.1. Phenotypic and morphological characterization provided cues for better drought adaptation in *cpm2*

It is a commonly observed phenomenon that overall plant growth is greatly affected by drought stress. Morphologically, shoot and the root system are most severely affected as both are the principle elements of plant adaptation to drought. In response to drought stress, plants generally limit the area of leaves in response to lower down the water budget spent in transpiration (Schuppler *et al.,* 1998). Since roots are the sole source to derive water from soil, the root growth, its density, proliferation and size could be the critical responses of plants to drought stress (Kavar *et al.,* 2007).

### 4.1.1. Less pronounced leaf rolling was perceived as a phenotypic signal of drought adaptation in *cpm2*

One of the well-known indicators for water stress is the rolling of leaves. Wenkert (1980) also reported that water stress in maize is indicated by loss of texture and development of

decoloration prior to leaf rolling. O' Toole and Cruz (1980) also reported that a good correlation exists between leaf rolling, stomatal resistance and leaf water potential in rice. Hence, leaf rolling can be used as an early symptom for drought susceptibility. Unexpectedly, *cpm2* showed a less drought-sensitive phenotype as compared to its WT background cultivar Nihonmasari. The finding that leaf rolling was less pronounced in *cpm2* correlated well with the fact that *cpm2* leaves remained turgid and fleshy as compared to the WT under both moderate and severe drought stress (Figure 9).

### 4.1.2 Roots of *cpm2* were better developed under both control and moderate drought stress

Serving as interfaces between plant and the soil, roots are the first organs to perceive and respond to drought (Trachsel *et al.,* 2010). The phenotype of the roots can therefore be considered as immediate manifestation for the ability of the plants to cope with drought stress. It has been reported in numerous studies previously that root architecture has a profound effect on overall growth and stress tolerance of crop plants including rice (Suji *et al.*, 2012, Trachsel *et al.,* 2011, Prince *et al.*, 2013). In drought tolerant genotypes, root architecture is greatly modified under drought stress (Osmont *et al.*, 2007). A more comprehensive root architecture analysis was performed on the two genotypes to gain deeper understanding of the factors affecting the phenotype. These studies further helped to determine whether root traits can be linked with better plant performance of *cpm2* under drought.

Deep rooting is a critical factor influencing the ability of plant to absorb water from the deeper layers of the soil (Franco *et al.*, 2006; 2011) Nodal roots are postembryonic roots, which arise from nodes at the base of the main stem and tillers. Functionally, nodal roots penetrate deeply into the soil and hence create a framework for whole root system to grow (Gowda *et al.*, 2011). Overall, this observation is also supported by a report by Manavalan *et al*. (2010) where they have shown that the distribution of roots, particularly those that can penetrate deeper in the soil, plays a crucial role in determining the ability of plants to capture key resources such as water and mobile nutrients like nitrate. *Cpm2* plants rooted deeper with more dry weight and had a longer nodal root length (Figure 13B & 10B).

In this study, longer lateral roots, increased total surface area and increased total root length were also observed in *cpm2* for control conditions, and these features were also maintained during moderate drought stress (Figure 10C). Lateral roots are basically the most active part of root system engaged in water uptake, and comprise the majority of length and surface area of the root system (Bauhus and Messier, 1999). This observation is consistent with similar observations reported by Rewald *et al.* (2011) where they found increased water uptake in citrus plants under moderate drought stress with increased lateral root length. Total root length represents the sum of the primary, crown, seminal and lateral roots (Li *et al*., 2015). It has been reported previously by Franco et al. (2008) that branching of the roots and total root length of *Silene vulgaris* plants were increased under moderate drought-stress. Root branching helps to circumvent localized water depletion around the roots, thus minimizing resistance to water transport into the root system and hence improved overall plant performance.

The JA mutant *cpm2* also showed increase in the fine root attributes (forks and tips) under control as well as under moderate stress stringency. Fine root attributes (≤2 mm diameter) play an important role in nutrient and water acquisition from soil to support the plants growth and survival (Pregitzer *et al.,* 2002; de Kroon, 2007). The increase in maximum root depth and root dry weight is thus an indicator of better plant resilience which probably contributes to sustenance under stress (Toorchi *et al.*, 2006; Kanbar *et al.,* 2009).
In this study, overall it was observed that the *cpm2* performed better in above mentioned root traits as compared to WT during moderate drought stress treatment.

### 4.2. Better physiological modulation in *cpm2* correlate with improved drought tolerance

Together with the rise in root mass particularly deeper in the soil and with the strategies that limit water loss - such as leaf rolling and stomatal closure - an improved plant water status could be maintained. These superior morpho-physiological adaptations are considered to be an important asset for improving drought tolerance in crops such as rice (Alsina *et al.,* 2007; Romero *et al.,* 2013)

### 4.2.1 Under drought, higher WUE in *cpm2* leads to better canopy production

Water Use Efficiency (WUE) is especially important trait in conditions where available water resources are limited or diminishing. The merits and disadvantages of various methods to estimate WUE have been discussed by Medrano *et al.* (2010), and also the choice of the appropriate method depends on the capacity, facilities, and scale of the specific study. Since *cpm2* is male sterile and because the absence of jasmonate renders it more susceptible to biotic stress (Riemann *et al.*, 2013), it was decided to evaluate WUE with respect to green leaf area at the seedling and early vegetative stage only. Importantly, in this study, with the increase in the severity of drought stress - the WUE increased in both the genotypes but altogether the mutant *cpm2* performed superior to WT under drought stress (Figure 14A). On the other hand, there was no significant difference under control conditions in both the genotypes (Figure 14 B). Better WUE of *cpm2* suggests better biomass production and this was also conserved by more developed aerial parts of the *cpm2* under drought stress.

### 4.2.2 Lower stomatal conductance in *cpm2* under drought: an effective water conservation strategy

Genotypes with increased stomatal conductance may in fact have a greater capacity to uptake available soil water via increased root area (Mitchell *et al.,* 1996) or osmotic adjustment (Blum, 2005), and therefore, can maintain transpiration during mild water stress conditions (Blum, 2009). However, increased stomatal conductance is a disadvantage when soil water deficits are more prevalent (Donovan *et al.,* 2007). Under severe drought, plants respond by lowering their stomatal conductance, whereas by a reduction of WUE plants exhaust available water faster, loose turgor, and eventually die if they do not succeed in a drought sustenance strategy (Donovan *et al.,* 2007). During our measurements, we also found interesting correlations supporting those mentioned by Mitchell *et al.* (1996) and Donovan *et al.* (2007): under moderate drought the WT showed a higher stomatal conductance (Figure 15A) whereas the *cpm2* responded more sensitively to drought stress and adjusted stomatal conductance (Figure 15B). But under severe stress, the conductance in the WT became very low which correlated well with its wilting phenotype as shown in Figure 9C, while the *cpm2* in similar conditions was performing better phenotypically due to a modest stomatal conductance as compared to WT.

### 4.2.3 Higher ABA levels in *cpm2* correlates with reduced stomatal conductance

During drought stress, ABA biosynthesis in the roots is known to be activated, and ABA is then translocated to the shoot via the xylem and can cause stomatal closure in the leaves (Gowing *et al.,* 1990; Zhang and Davies, 1990; Gomes *et al.,* 1997). As stomatal conductance was regulated differently in the *cpm2*, it could be predicted that abscisic acid (ABA), the main hormone controlling stomatal aperture, could be regulated differentially. In fact, the different accumulation of ABA in WT and *cpm2* correlated well with the differences of stomatal conductance(Figure 16A).

Analysis of morpho-physiological traits leads to the following scenario, explaining, why the mutant performs better under drought stress at physiological level. This improved performance is linked with a swifter adjustment of stomatal conductance appropriate to the respective level of stress stringency, correlated with corresponding modulations of ABA accumulation in the shoots. Moreover, the better overall root architecture under control conditions already, provides the *cpm2* with the ability to scavenge for water and nutrients even under drought. This allows *cpm2* to buffer the initially moderate drought stress more efficiently, as seen from the superior control of stomatal conductance as compared to the WT. When the conditions become too adverse, under severe stress, *cpm2* once is still able to adapt by increasing the content of ABA hormone, which leads to lower stomatal conductance and better WUE, whereas the WT seemed to have lost any control over the water status.

## 4.3. Functional context of the protein candidates as revealed by the TMT approach

As it is well evident that genome and transcriptome expression profiling do not accurately relate with proteome complexity (Maier, 2009) hence comparative proteomics has been successfully employed to identify proteins that are differentially regulated proteins in response to salt (Pandhal *et al.,*2008) and drought stress (Hajheidari *et al.* 2005). The drawback of proteomics has been the difficulty to standardize 2D electrophoresis to a state, that biological replicas yield the same patterns. The highly sensitive proteomic platform based on the isobaric labels tandem mass tags (TMT) has therefore been developed to improve reliability by a mulitplexing strategy and has meanwhile emerged as one of the

most robust proteomics techniques (Thompson *et al.*, 2003; Pagel *et al.*, 2015). In this study, TMT approach was applied for the first time ever to dissect the root proteome of a JA deficient mutant of rice. Since the morpho-physiological analysis indicated that the superior drought tolerance of the jasmonate deficient rice mutant was located in the root, a targeted TMT strategy was utilized to identify the key molecular players in the roots. As a logical filter to prioritize the hits, the comparison was done with the respective WT background, and detailed knowledge on the temporal dynamics of drought responses in WT and mutant was utilized for the data analysis. This approach should allow us to specifically unravel insights into the molecular mechanisms associated with drought stress in a jasmonate dependent and/or independent manner.

The data obtained through this root proteome analysis contributed for a proteomic explanation for the differential adaptive morpho-physiological responses observed among the WT and *cpm2* during the drought stress - which are described in the previous sections.

### 4.3.1 Metabolism related DEPs contributed to enhanced drought tolerance in *cpm2*

During plant adaptation to abiotic stress, a change in glycolysis and gluconeogenesis is considered to be a normal trend. Glycolysis is an important metabolic pathway in carbohydrate metabolism, and drought stress leads to altered sucrose and amino acid contents, which was revealed by metabolite analysis (Broeckling *et al.,* 2005). During the root proteome analysis, three important proteins involved in glycolysis were differentially expressed under drought stress and they were abundant in *cpm2*: 1) Triose Phosphate Isomerase (TPI) 2) glyceraldehyde phosphate dehydrogenase (G3PD) and 3) pyruvate decarboxylase (PDC) (Figure 19). G3PD may provide a direct connection between membrane lipid–based signaling, energy metabolism and growth control in a plant's response to ROS and water stress (Guo *et al.,* 2012). TPI has also been reported to be involved in plant stress response and its expression is also induced in response to water deficit conditions in maize (Riccardi *et al.*, 1998). TPI was also found to be more abundant in *cpm2* roots. PDC is the first and key enzyme of ethanolic fermentation, which branches off the main glycolytic pathway at pyruvate (Zabalza *et al.,* 2009). Recent research indicates that ethanolic fermentation occurs not only under anaerobic conditions but also under aerobic conditions, taking part in carbohydrate and energy metabolism (Chen and Han, 2011). In *Arabidopsis*, transgenic and mutant experiments indicated that PDC1 and PDC2 are important for the improved survival

of roots and leaves under low-oxygen conditions (Mithran *et al.,* 2014). Thus, it appears that ethanolic fermentation might be an important switch in regulating carbohydrate metabolism under stress conditions such as drought.

Protein synthesis is of critical importance for plant abiotic stress adaption. The levels of many components of the protein synthesis machinery are altered under abiotic stress conditions such as salinity and drought. Ribosomal proteins, are also an important component of protein synthesis machinery, are drought induced in *Arabidopsis* and maize roots (Ghosh *et al.,* 2014). In the present study, two DEPs related to peptidase T1 family were found to be abundant in *cpm2* (Figure 20). Similarly, one 60 S ribosomal protein was also differentially expressed and was more abundant in *cpm2* (Figure 20). This suggested that protein synthesis machinery might be upregulated in *cpm2* which seems to help the plant to battle drought stress.

### 4.3.2 Better cellular homeostasis and increased nitrogen metabolism: *cpm2* more tolerant against drought stress

Water uptake and its flow across the cell membrane are essential for plant growth and sustenance under normal and stressful conditions. The plasma membrane intrinsic protein (PIP) is a subfamily of aquaporins comprising two subgroups of PIP1 and PIP2; and PIP2 proteins show higher water channel activity (Chaumont *et al.*, 2000). However, under drought stress a strong down-regulation of PIP genes transcription was also observed in the roots and twigs of olive plants (Sechhi *et al.,* 2007), and in the roots of tobacco (Mahdieh *et al.,* 2008). It is a general observation that down-regulation of some of the PIP genes is believed to inhibit water loss and to help prevent backflow of water to dried soil (Afzal *et al.,* 2016). In the present study, abundance of one such DEP; OsPIP1 in WT and its less abundance in *cpm2* (Figure 21) was probably to resist the rapid diffusion of water to the outside of plasma membrane and this can help to maintain cellular homeostasis under drought in the case c*pm2*.

3-isopropylmalate dehydrogenase was also identified as a DEP, which is involved in leucine biosynthesis, in *cpm2* as an abundant protein (Figure 21). Leucine is an important amino acid and hence an important structural component of many proteins in plants.

For plants, nitrogen is an essential nutrient and a critical limiting factor in plant productivity. In plants, all inorganic nitrogen is first converted to ammonium before it could be utilized for biosynthesis of organic compounds. Glutamine Synthetase (GS) then converts ammonium into glutamine, which supplies nitrogen groups, either directly or via glutamate, for almost every nitrogenous cell compounds. Thus, being the first enzyme of the nitrogen assimilatory pathway, GS is believed to play a regulatory role in nitrogen metabolism and plant productivity (Lea and Miffin, 2010). In this study, GS was identified as a DEP and it was highly abundant in *cpm2* (Figure 21). It is widely believed that increased flux through N metabolic pathways suggests an enhancement of N uptake by the roots under stress, which is important for plant stress tolerance. Also, according to Kalamaki *et al.* (2009) increased expression of GS genes contributes to drought and salt tolerance.

### 4.3.3 Less cellular oxidative damage makes *cpm2* more sustainable against drought stress.

Much of the injury to plants caused by various stressful conditions is frequently due to oxidative damage at the cellular level, which results from increased production of reactive oxygen species (ROS) (Sharma *et al.*, 2012). Higher plants have developed a complex series of detoxification mechanisms to tightly control the level of ROS through enzymatic and non-enzymatic approaches. Ascorbate (AsA) and glutathione (GSH), non-enzymatic antioxidants are crucial for plant defense against oxidative stress, playing a key role as antioxidant buffers (Foyer and Noctor, 2005). In this study, two of these Glutathione-S-Transferase(GST) were found to be as Differentially Expressed Proteins (DEP), OsGSTF2 and OsGSTU12 and they were considerably highly abundant in *cpm2* (Figure 24). GSTs are best known for the detoxification of xenobiotics but they can also act as antioxidants by tagging oxidative degradation products (especially from fatty acids and nucleic acids) for removal or by acting as a glutathione peroxidase to directly scavenge peroxides (Frova, 2003). It has been widely reported that ascorbate peroxidase (APX) participate in the ascorbate-glutathione cycle, which is an important process for free radical detoxification (Cramer *et al.,* 2013). Significant abundance of OsAPX7 was identified in *cpm2* (Figure 24); therefore, it could be speculated that APX activity may influence drought tolerance by regulating glycerophospholipid metabolism and the ascorbate pathway in *cpm2* for better drought adaptation. Similarly, OsSHMT2 was more abundant in *cpm2.* Serine hydroxymethyltransferases (SHMTs) are

known to be part of dissipative mechanisms to minimize production of ROS under biotic and abiotic conditions in plants (Morena *et al.,* 2005).

### 4.3.4 Enhanced cell organization and augmented cell wall adaptations promoted drought tolerance in *cpm2* roots

Several proteins identified as differentially expressed between the two genotypes indicated the capacity of the *cpm2* to make the necessary favourable cell wall adaptation in the roots against drought stress. Proteins such as tubulins, profilins, glycosyl hydrolases, O-methyl transferases, glycosyl hydrolases and UDP-D-Glucose dehydrogenase were able to shed some light on the morphological adaptations in *cpm2.*

Glycosyl hydrolases plays an important role in the formation of branched glucans, as well as in cell-wall assembly and rearrangement. The biochemical activity of these enzymes is based on the hydrolytic cleavage of 1,3-D-glucosidic linkages between β-1,3-glucans, which are major components of the cell wall surrounding fungi and plants. The enzymatic activity of β-1,3-glucanases is crucial in the chemical changes of the glucan composition and in the remodelling of the cell wall (Torres *et al.,* 2015). In this study, one such glycosyl hydrolase was more abundant in *cpm2* (Figure 25). Although not much is known about its exact function in drought but it is believed to have a critical role in cell wall remodelling which is a critical adaptation during various abiotic stresses.

Plant O-Methyltransferases (OMT) are multifunctional enzymes that have varied functions ranging from their role in cell wall adaptations as well as they catalyze *O*-methylation of multiple secondary metabolites that are involved in diverse biological processes such as plant growth, development, and environmental responses. There is some evidence that OMT genes may be involved in various abiotic and biotic stress responses (Barakat *et al.,* 2011). One such OMT was identified as a DEP, and it was more abundant in *cpm2* (Figure 25).

UDP-D-glucose dehydrogenase (UDPGDH) oxidizes UDP-Glc (UDP-d-glucose) to UDP-GlcA (UDP-d-glucuronate), the precursor of UDP-D-xylose and UDP-L-arabinose which are major cell wall polysaccharide precursors (Kärkönen *et al.*, 2005a). An increased relative abundance of UDP-glucose 6-dehydrogenase (UGPGDH) was observed in *cpm2* (Figure 25) which may indicate enhanced synthesis of pectins and hemicelluloses, as well as the remodeling of cell walls in response to stress (Kärkönen *et al.,* 2005b). This is also in agreement by a report by

Yoshimura *et al.* (2008) where they also reported UDPGDH been accumulating under drought stress in watermelon roots.

β-Tubulins are major components of the microtubules that are involved in many cellular processes, such as cell division and intracellular transport in eukaryotic organisms (Zhao *et al.,* 2014). β-Tubulin1 was found to be more abundant in *cpm2* (Figure 26).

It has been reported by Ramachandran *et al.* (2000) that actin nucleation protein plays a role in cell elongation, cell shape maintenance, and polarized growth of root hair. One such actin nucleation protein was also more abundant in *cpm2* (Figure 26). Though for both these proteins, no direct role in drought has been implicated in the past as per available information but it is assumed that they have an important role to play in organization of cytoskeleton which is very necessary for cell elongation and cell shape maintenance in adverse conditions such as drought.

Lignins also form an important structural part of plant cell walls. Reports have shown that there was an increased expression of genes involved in lignin biosynthesis during the intermediate and final stages of water stress (from 48 h to 72 h) in rice roots (Oryza sativa L.), such as those coding for PAL, C3H, 4-coumarate: coenzyme A ligase (4CL), caffeoyl coenzyme A O-methyltransferase (CCoAOMT), cinnamyl alcohol dehydrogenase (CAD), and peroxidase (Yang *et al.*, 2006). Similarly, lignin synthesis-related proteins were abundant mostly in the later stage of drought stress in the roots of wild watermelon (*Citrullus lanatus* sp.), which may function in the enhancement of physical desiccation tolerance and drought adaptation (Yoshimura *et al.,* 2008). In this study as well, several enzymes involved in the phenylpropanoid pathway were found to be specifically abundant in the *cpm2* (Figure 27). In this study, two PALs were found unique and abundant in *cpm2*. In addition, 4CL was also unique and abundant in *cpm2*. Also, other important proteins for COMT and CAD were more abundant in *cpm2*. The proteomic abundances of these important enzymes of phenylpropanoid pathway also co-related well with the transcript abundances much specifically during moderate stress (Figure 28, 29, 30).

### 4.4 Models explaining for mechanisms of enhanced drought adaptation in *cpm2*

#### A. Importance to constrain OPDA to suppress drought induced senescence

In this study, ALLENE OXIDE CYCLASE (AOC) was found to be unique to WT and highly abundant under drought (Figure 22A). This reconfirmed the lack of AOC in *cpm2*. Another protein, 12-OXOPHYTODIENOATE REDUCTASE (OPR7), which is downstream of AOC in the JA biosynthesis pathway, was more abundant in *cpm2* under drought (Figure 23A). However, when comparing OPDA and JA hormonal levels in plants grown in soil under greenhouse conditions with similar treatment of moderate and severe stress respectively - no significant differences were found due to the refractory effect of drought stress. As hormonal levels are being transient it is possible to miss those precise time points during which alterations in their trace amounts could be detected. Due to practical limitations, it was almost impossible to repeat these hormonal measurements as determining the precise time points under these natural conditions were cumbersome as well as time and resource consuming. However based on the proteomic evidence, it can be speculated that in WT because of very high abundance of AOC and less abundance of downstream OPR7 respectively, leads to an increased OPDA accumulation. This speculation also becomes consistent with the findings of Hazman *et al.* (2015) using *cpm2* and WT, where increased OPDA accumulation in WT was perceived as a damage signal during salinity stress. However to confirm this finding during drought at the metabolite level, further measurements will be required to confirm the OPDA accumulation.

#### B. Cell wall rigidification could have improved mechanical penetrance of *cpm2* root under drought stress

Root traits are known to be important for better plant sustenance under drought stress. The structure and development of root system largely determines plant function under drought. Roots rapidly sense changes in water potential and significantly alter root architecture and intensify cell wall rigidification in an attempt to acquire more water and for improved tissue water status simultaneously to maintain non-detrimental water potential. Among the very first cellular response to drought stress is cell wall hardening, which relates to the reduced plastic extensibility and increased elastic modulus of the cell wall. This wall hardening is biochemically related to diminished cell wall acidification and increased cross linking by

phenolic substances such as lignins (Fan and Neumann, 2004; Fan *et al.,* 2006). Moreover, cell wall hardening with increased energy metabolism in the apical regions at the same time translates into enhanced growth which may lead to deeper root penetration into the soil pans where the soil moisture contents are considerably higher. In case of *cpm2* roots, this phenomenon correlates very well as overall; cell wall metabolism was higher together with a better root architecture which renders better sustenance during drought stress.

**C. Growth-Defence tradeoffs: better plant performance in case of *cpm2***

Implementation of defence responses in plants inflicts a considerable requirement of resources, which has been suggested to reduce growth. This hypothesis is based on the notion that being well-contended (i.e., having strong, prior defensive mechanisms) may not always be the best fitting defence strategy, probably because allotment of metabolites and proteins for resistance may curb other plant physiological processes (Kempel *et al.,* 2011). Also, this negative impact on growth could result from diminished leaf area (or photosynthesis) which could reduce the overall pool of energy reserves, and/or a diversion of resources away from growth and more towards defence in a serious detrimental condition such as drought.

It has been shown long back that activation of JA signalling by applying JA into the growth medium results in growth inhibition (Staswick *et al.,* 1992). It is also known that suppressing components in JA-mediated defence signalling alleviates fitness costs observed in wild-type plants (Meldau *et al.,* 2012). In the case of constitutive defence response, diminished fitness could be due to redundant deviation of energy reserves away from growth in the absence of stress. Based on these evidences, it could be predicted that in *cpm2* under drought stress, there exists a defence-growth trade-off, which may shift the whole physiological phenomenon towards superior plant performance under drought as already observed in *cpm2*. In particular, Gibberellic acid (GA), a known growth hormone - which also has a function during abiotic stress tolerance, may be involved in growth promotion (Colebrook *et al.,* 2014). Recent studies have shown an important role for JA–GA signaling crosstalk in regulating the growth–defence trade-off. In *Nicotiana attenuata*, an increased JA level has a negative effect on GA biosynthesis in stems resulting in growth inhibition (Heinrich *et al.,* 2013). Conversely, in several *Arabidopsis* mutants in which the DELLA transcriptional repressors are stabilized, MYC2-dependent JA-responsive genes were hypersensitive to JA

treatment resulting in increased growth inhibition (Hou *et al.,* 2010). This JA-GA antagonistic relationship, in *cpm2,* can be predicted to be conferring increased growth due to increased induction of GA signalling under drought.

**4.5 Concluding Remarks:**

Till date only a handful of reports have been able to show a co-relation between JA functioning as a stress hormone in drought tolerance and that too mainly in *Arabidopsis* but not in model crop species such as rice under realistic drought conditions. In order to assess the role of JA during drought stress in rice, this whole study was formulated using the *cpm2*, which is a JA deficient mutant. Firstly, the comparative changes between WT and *cpm2* in some morpho- physiological traits under realistic drought stress conditions were analysed. The mutant, *cpm2* showed morpho-physiological adaptations to drought, for instance *cpm2* had lower stomatal conductance and higher water use efficiency under drought as compared to the WT with better developed root architecture. As roots are considered as the principal organs to perceive low water potential in the soil, a targeted root proteome analysis was undertaken using high throughput Tandem Mass Tag (TMT) approach. It was observed that the biological processes were diverse in the sense that cellular metabolic pathways, communication between cells, and the processes involved in stress protective roles were affected by drought stress and were upregulated in the jasmonate mutant *cpm2*. As a central participant of the whole story, based on the proteomic evidences, it is postulated that in *cpm2* suppression of OPDA accumulation due to the loss of AOC represses the drought induced senescence. Moreover, a hormonal cross-talk could be implicated as a major player in regulating trade-offs needed to achieve a balance between growth and defence in *cpm2* during drought stress. Based on all these results, it can be suggested that JA signalling might negatively influence drought tolerance by orchestrating a block on critical morpho-physiological and molecular changes necessary for stress adaptation.

These revelations could provide us powerful tools for improving drought tolerance in plants and also to develop new drought tolerant varieties through smart breeding in the future. However, on the other hand it will be a tedious task for molecular biologists to manipulate JA biosynthesis or signalling in such a manner so that negative side effects commonly associated with reduced jasmonate function such as reduced fertility and enhanced sensitivity to pathogens does not arise. Unravelling the critical nodes in the JA biosynthetic

pathway and its fine-tuning could be useful for stress tolerance without the associated penalties will largely denote the extent of success in employing these strategies for breeding stress tolerant crop varieties

## 5. Outlook

In future, further gene validation through genetic engineering is recommended in order to provide conclusive evidence on contribution of above mentioned pathways to drought tolerance trait in rice. This is because they exhibited differential expression between WT and *cpm2* under control and drought treatment. For instance genes that provide insights into pathways regulating root architecture and/or secondary metabolism could be used to produce plants with enhanced water usage efficiency and higher drought tolerance through genetic engineering.

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## Suppl Table 1. List of unique proteins - more abundant in WT

| Locus ID | Protein name | Gene Ontology | Stress/Control Log Ratio |
|---|---|---|---|
| loc_os01g41710 | chlorophyll A-B binding protein, putative, expressed | PS.light reaction.photosystem II.LHC-II | 1.352 |
| loc_os06g04270 | transketolase, chloroplast precursor, putative, expressed | PS.calvin cycle.transketolase | 1.181 |
| | | **major CHO metabolism** | |
| loc_os08g20660 | sucrose-phosphate synthase, putative, expressed | major CHO metabolism.synthesis.sucrose.SPS | 1.155 |
| loc_os05g50380 | glucose-1-phosphate adenylyltransferase large subunit, chloroplast precursor, putative, expressed | major CHO metabolism.synthesis.starch.AGPase | 1.354 |
| | | **glycolysis** | |
| loc_os03g56460 | glucose-6-phosphate isomerase, putative, expressed | glycolysis.plastid branch.glucose-6-phosphate isomerase | 1.173 |
| loc_os01g09570 | 6-phosphofructokinase, putative, expressed | glycolysis.plastid branch.phosphofructokinase (PFK) | 1.261 |
| | | **fermentation** | |
| loc_os09g26880 | aldehyde dehydrogenase, putative, expressed | fermentation.LDH | 1.117 |
| | | **OPP** | |
| loc_os07g22350 | glucose-6-phosphate 1-dehydrogenase, chloroplast precursor, putative, expressed | OPP.oxidative PP.G6PD | 1.014 |
| | | **TCA / org. transformation** | |
| loc_os08g33440 | 2-oxo acid dehydrogenases acyltransferase domain containing protein, expressed | TCA / org. transformation.TCA.pyruvate DH.E2 | 1.022 |
| loc_os06g01630 | 2-oxo acid dehydrogenases acyltransferase domain containing protein, expressed | TCA / org. transformation.TCA.pyruvate DH.E2 | 1.055 |
| loc_os04g32330 | dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial precursor, putative, expressed | TCA / org. transformation.TCA.2-oxoglutarate dehydrogenase | 1.312 |
| loc_os07g04240 | succinate dehydrogenase flavoprotein subunit,mitochondrial precursor, putative, expressed | TCA / org. transformation.TCA.succinate dehydrogenase | 1.392 |
| loc_os02g03260 | 3-isopropylmalate dehydratase large subunit 2, putative, expressed | TCA / org. transformation.other organic acid transformaitons.aconitase | 1 |
| loc_os01g19450 | ATP-citrate synthase subunit 1, putative, expressed | TCA / org. transformation.other organic acid transformaitons.atp-citrate lyase | 1.031 |
| | | **cell wall** | |
| loc_os08g03570 | NAD dependent epimerase/dehydratase family protein, putative, expressed | cell wall.precursor synthesis.MUR4 | 1.036 |
| loc_os01g47780 | fasciclin domain containing protein, expressed | cell wall.cell wall proteins.AGPs.AGP | 1.019 |
| loc_os07g07990 | uncharacterized protein At4g06744 precursor, putative, expressed | cell wall.cell wall proteins.LRR | 1.255 |
| loc_os11g43750 | polygalacturonase, putative, expressed | cell wall.degradation.pectate lyases and polygalacturonases | 1.612 |
| loc_os10g40700 | expansin precursor, putative, expressed | cell wall.modification | 1.305 |
| | | **lipid metabolism** | |
| loc_os08g39350 | glycerophosphoryl diester phosphodiesterase family protein, putative, expressed | lipid metabolism.lipid degradation.lysophospholipases.glycerophosphodiester phosphodiesterase | 1.073 |
| loc_os02g17390 | 3-hydroxyacyl-CoA dehydrogenase, putative, expressed | lipid metabolism.lipid degradation.beta-oxidation.multifunctional | 1.08 |
| | | **N-metabolism** | |
| loc_os01g48960 | glutamate synthase, chloroplast precursor, putative, expressed | N-metabolism.ammonia metabolism.glutamate synthase | 1.356 |
| | | **amino acid metabolism** | |
| loc_os05g47640 | threonine synthase, chloroplast precursor, putative, expressed | amino acid metabolism.synthesis.aspartate family.threonine.threonine synthase | 1.227 |
| loc_os02g24020 | dihydrodipicolinate reductase, putative, | amino acid metabolism.synthesis.aspartate | 1.012 |

| | | | |
|---|---|---|---|
| | expressed | family.lysine.dihydrodipicolinate reductase | |
| loc_os03g42110 | semialdehyde dehydrogenase, NAD binding domain containing protein, putative, expressed | amino acid metabolism.synthesis.aspartate family.misc.homoserine.aspartate semialdehyde dehydrogenase | 1.118 |
| loc_os12g04440 | 2-isopropylmalate synthase B, putative, expressed | amino acid metabolism.synthesis.branched chain group.leucine specific.2-isopropylmalate synthase | 1.209 |
| loc_os04g55720 | D-3-phosphoglycerate dehydrogenase, chloroplast precursor, putative, expressed | amino acid metabolism.synthesis.serine-glycine-cysteine group.serine.phosphoglycerate dehydrogenase | 1.086 |
| loc_os03g27230 | phospho-2-dehydro-3-deoxyheptonate aldolase, chloroplast precursor, putative, expressed | amino acid metabolism.synthesis.aromatic aa.chorismate.3-deoxy-D-arabino-heptulosonate 7-phosphate synthase | 1.205 |
| loc_os06g42560 | tryptophan synthase beta chain 2, putative, expressed | amino acid metabolism.synthesis.aromatic aa.tryptophan.tryptophan synthase | 1.173 |
| loc_os01g13190 | histidinol dehydrogenase, chloroplast precursor, putative, expressed | amino acid metabolism.synthesis.histidine | 1.417 |
| loc_os06g04380 | aminomethyltransferase, putative, expressed | amino acid metabolism.degradation.serine-glycine-cysteine group.glycine | 1.333 |
| loc_os04g57410 | methylthioribose kinase, putative, expressed | amino acid metabolism.misc | 1.016 |
| | | **S-assimilation** | |
| loc_os04g02050 | bifunctional 3-phosphoadenosine 5-phosphosulfate synthetase, putative, expressed | S-assimilation.ATPS | 1.206 |
| | | **secondary metabolism** | |
| loc_os02g03260 | 3-isopropylmalate dehydratase large subunit 2, putative, expressed | secondary metabolism.sulfur-containing.glucosinolates.synthesis.aliphatic.methylthio alkylmalate isomerase large subunit (MAM-IL) | 1 |
| | | **hormone metabolism** | |
| loc_os07g14610 | IAA-amino acid hydrolase ILR1-like 6 precursor, putative, expressed | hormone metabolism.auxin.synthesis-degradation | 1 |
| loc_os01g43090 | oxidoreductase, aldo/keto reductase family protein, putative, expressed | hormone metabolism.auxin.induced-regulated-responsive-activated | 1.001 |
| loc_os03g32314 | allene oxide cyclase 4, chloroplast precursor, putative, expressed | hormone metabolism.jasmonate.synthesis-degradation.allene oxidase cyclase | 7.52 |
| | | **Co-factor and vitamine metabolism** | |
| loc_os08g37605 | riboflavin biosynthesis protein ribAB, chloroplast precursor, putative, expressed | Co-factor and vitamine metabolism.riboflavin.GTP cyclohydrolase II | 1.332 |
| | | **redox** | |
| loc_os03g13160 | non-symbiotic hemoglobin 2, putative, expressed | redox.heme | 1.167 |
| loc_os03g03910 | catalase domain containing protein, expressed | redox.dismutases and catalases | 1.09 |
| loc_os02g02400 | catalase isozyme A, putative, expressed | redox.dismutases and catalases | 1.088 |
| | | **nucleotide metabolism** | |
| loc_os02g50350 | dihydroorotate dihydrogenase protein, putative, expressed | nucleotide metabolism.degradation.pyrimidine.dihydrouracil dehydrogenase | 1.064 |
| loc_os11g20790 | adenylate kinase, putative, expressed | nucleotide metabolism.phosphotransfer and pyrophosphatases.adenylate kinase | 1.046 |
| | | **Biodegradation of Xenobiotics** | |
| loc_os05g22970 | glyoxalase family protein, putative, expressed | Biodegradation of Xenobiotics | 1.515 |
| | | **misc** | |
| loc_os01g69130 | dynamin family protein, putative, expressed | misc.dynamin | 1.15 |
| loc_os06g13820 | dynamin, putative, expressed | misc.dynamin | 1.131 |
| | | **RNA** | |
| loc_os03g59050 | DEAD-box ATP-dependent RNA helicase, putative, expressed | RNA.processing.RNA helicase | 1.102 |
| loc_os07g31270 | cupin 2, conserved barrel domain protein, putative, expressed | RNA.regulation of transcription.Trihelix, Triple-Helix transcription factor family | 1.208 |
| loc_os01g14440 | WRKY1, expressed | RNA.regulation of transcription.WRKY domain transcription factor family | 1.06 |
| loc_os02g32350 | TUDOR protein with multiple SNc domains, putative, expressed | RNA.regulation of transcription.Zn-finger(CCHC) | 1.078 |

| | | | |
|---|---|---|---|
| loc_os07g46480 | eukaryotic aspartyl protease domain containing protein, expressed | RNA.regulation of transcription.unclassified | 1.071 |
| loc_os10g41440 | KH domain containing protein, putative, expressed | RNA.RNA binding | 1.049 |
| loc_os11g41890 | RNA recognition motif containing protein, putative, expressed | RNA.RNA binding | 1.253 |
| loc_os07g39560 | RNA recognition motif containing protein, putative, expressed | RNA.RNA binding | 1.046 |
| | | **DNA** | |
| loc_os01g36090 | DNA-damage-repair/toleration protein DRT102, putative, expressed | DNA.repair | 1.034 |
| | | **protein** | |
| loc_os08g42560 | tRNA synthetase class II core domain containing protein, expressed | protein.aa activation.glycine-tRNA ligase | 1.128 |
| loc_os07g05580 | ribosomal protein L7Ae, putative, expressed | protein.synthesis.ribosomal protein.eukaryotic.40S subunit.S12 | 1.07 |
| loc_os06g04290 | S10/S20 domain containing ribosomal protein, putative, expressed | protein.synthesis.ribosomal protein.eukaryotic.40S subunit.S20 | 1.16 |
| loc_os07g42450 | ribosomal protein S2, putative, expressed | protein.synthesis.ribosomal protein.eukaryotic.40S subunit.SA | 1.032 |
| loc_os01g47660 | 60S ribosomal protein L18a, putative, expressed | protein.synthesis.ribosomal protein.eukaryotic.60S subunit.L18A | 1.311 |
| loc_os01g71090 | xylanase inhibitor, putative, expressed | protein.degradation.aspartate protease | 1.785 |
| loc_os05g49200 | aspartic proteinase oryzasin-1 precursor, putative, expressed | protein.degradation.aspartate protease | 1.257 |
| loc_os12g13390 | aspartyl aminopeptidase, putative, expressed | protein.degradation.aspartate protease | 1.072 |
| loc_os05g33430 | xyloglucanase inhibitor, putative, expressed | protein.degradation.aspartate protease | 1.06 |
| loc_os06g20040 | aspartic proteinase nepenthesin-2 precursor, putative, expressed | protein.degradation.aspartate protease | 1.049 |
| loc_os05g44310 | vesicle-fusing ATPase, putative, expressed | protein.degradation.AAA type | 1.045 |
| loc_os11g01510 | ubiquitin-activating enzyme, putative, expressed | protein.degradation.ubiquitin.E1 | 1.045 |
| loc_os01g36930 | ubiquitin carboxyl-terminal hydrolase 6, putative, expressed | protein.degradation.ubiquitin.ubiquitin protease | 1.15 |
| loc_os01g08200 | ubiquitin carboxyl-terminal hydrolase 14, putative, expressed | protein.degradation.ubiquitin.ubiquitin protease | 1.448 |
| loc_os05g02510 | beta-hexosaminidase precursor, putative, expressed | protein.glycosylation.alpha-1,3-mannosyl-glycoprotein-beta-1,2-N-acetylglucosaminyltransferase(GnTI) | 1.11 |
| loc_os05g02510 | beta-hexosaminidase precursor, putative, expressed | protein.glycosylation.alpha-1,6-mannosyl-glycoprotein-beta-1,2-N-acetylglucosaminyltransferase(GnTII) | 1.11 |
| | | **signalling** | |
| loc_os11g31530 | BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor, putative, expressed | signalling.receptor kinases.leucine rich repeat XI | 1.048 |
| | | **transport** | |
| loc_os01g46980 | vacuolar ATP synthase subunit E, putative, expressed | transport.p- and v-ATPases.H+-transporting two-sector ATPase.subunit E | 1.139 |
| loc_os06g21920 | inorganic phosphate transporter 1-9, putative, expressed | transport.phosphate | 1.011 |

## Suppl Table 2: List of unique proteins - more abundant in *cpm2*

| Locus ID | Protein name | Gene Ontology | Stress/control log ratio |
|---|---|---|---|
| | | **PS** | |
| loc_os04g53210 | hydroxyacid oxidase 1, putative, expressed | PS.photorespiration.glycolate oxidase | 1.575 |
| loc_os01g05490 | triosephosphate isomerase, cytosolic, putative, expressed | PS.calvin cycle.TPI | 1.904 |
| loc_os09g36450 | triosephosphate isomerase, chloroplast precursor, putative, expressed | PS.calvin cycle.TPI | 1.233 |
| loc_os01g67860 | fructose-bisphospate aldolase isozyme, putative, expressed | PS.calvin cycle.aldolase | 1.129 |
| loc_os01g02880 | fructose-bisphospate aldolase isozyme, putative, expressed | PS.calvin cycle.aldolase | 1.164 |
| loc_os05g33380 | fructose-bisphospate aldolase isozyme, putative, expressed | PS.calvin cycle.aldolase | 1.737 |
| | | **Major CHO Metabolism** | |
| loc_os02g05030.1 | sucrose-phosphatase, putative, expressed | major CHO metabolism.synthesis.sucrose.SPP | 1.007 |
| | | **Minor CHO Metabolism** | |
| loc_os10g22450 | inositol-3-phosphate synthase, putative, expressed | minor CHO metabolism.myo-inositol.InsP Synthases | 1.652 |
| loc_os07g09330 | inositol-1-monophosphatase, putative, expressed | minor CHO metabolism.myo-inositol.inositol phosphatase | 1.037 |
| loc_os04g56290 | aldose 1-epimerase, putative, expressed | minor CHO metabolism.others | 1.037 |
| loc_os05g39690 | oxidoreductase, aldo/keto reductase family protein, putative, expressed | minor CHO metabolism.others | 1.359 |
| loc_os11g29370 | haloacid dehalogenase-like hydrolase family protein, putative, expressed | minor CHO metabolism.others | 1.008 |
| loc_os07g48160 | alpha-galactosidase precursor, putative, expressed | minor CHO metabolism.galactose.alpha-galactosidases | 1.163 |
| | | **Gluconeogenesis** | |
| loc_os02g13840 | citrate synthase, putative, expressed | gluconeogenese/ glyoxylate cycle.citrate synthase | 1.195 |
| loc_os08g33720 | malate lactate/malate dehydrogenase, putative, expressed | gluconeogenesis.Malate DH | 1.078 |
| | | **TCA** | |
| loc_os05g49880 | lactate/malate dehydrogenase, putative, expressed | TCA / org. transformation.TCA.malate DH | 1.514 |
| loc_os01g46070 | lactate/malate dehydrogenase, putative, expressed | TCA / org. transformation.TCA.malate DH | 1.255 |
| loc_os08g33720 | lactate/malate dehydrogenase, putative, expressed | TCA / org. transformation.TCA.malate DH | 1.078 |
| loc_os10g33800 | lactate/malate dehydrogenase, putative, expressed | TCA / org. transformation.other organic acid transformaitons.cyt MDH | 1.392 |
| | | **mitochondrial electron transport / ATP synthesis** | |
| loc_os02g30460 | uncharacterized protein MJ0304, putative, expressed | mitochondrial electron transport / ATP synthesis.NADH-DH.complex I.carbonic anhydrase | 1 |
| | | **Cell Wall** | |
| loc_os03g16980 | NAD dependent epimerase/dehydratase family domain containing protein, expressed | cell wall.precursor synthesis.UXS | 1.179 |
| loc_os04g56520.1 | alpha-1,4-glucan-protein synthase, putative, expressed | cell wall.cell wall proteins.RGP | 1.255 |
| | | **Lipid Metabolism** | |
| loc_os08g23810 | enoyl-acyl-carrier-protein reductase NADH, chloroplast precursor, expressed | lipid metabolism.FA synthesis and FA elongation.enoyl ACP reductase | 1.052 |
| loc_os11g10980 | pyruvate kinase, putative, expressed | lipid metabolism.FA synthesis and FA elongation.pyruvate kinase | 1.252 |
| loc_os01g07760 | phospholipase D, putative, expressed | lipid metabolism.lipid degradation.lysophospholipases.phospholipase D | 1.024 |
| | | **N-metabolism** | |

| | | | |
|---|---|---|---|
| loc_os03g58040 | glutamate dehydrogenase protein, putative, expressed | N-metabolism.N-degradation.glutamate dehydrogenase | 1.099 |
| | | **Amino acid Metabolism** | |
| loc_os03g19280 | argininosuccinate lyase, putative, expressed | amino acid metabolism.synthesis.glutamate family.arginine.argininosuccinate lyase | 1.166 |
| loc_os03g50510 | threonine dehydratase biosynthetic, chloroplast precursor, putative, expressed | amino acid metabolism.synthesis.branched chain group.isoleucine specific.threonine ammonia-lyase | 1.007 |
| loc_os12g42980 | cysteine synthase, putative, expressed | amino acid metabolism.synthesis.serine-glycine-cysteine group.cysteine.OASTL | 1.06 |
| loc_os06g04280 | 3-phosphoshikimate 1-carboxyvinyltransferase, chloroplast precursor, putative, expressed | amino acid metabolism.synthesis.aromatic aa.chorismate.5-enolpyruvylshikimate-3-phosphate synthase | 1.665 |
| loc_os02g57260 | 3-ketoacyl-CoA thiolase, peroxisomal precursor, putative, expressed | amino acid metabolism.degradation.branched-chain group.shared | 1.472 |
| | | **Secondary Metabolism** | |
| loc_os09g07830 | acetyl-CoA acetyltransferase, cytosolic, putative, expressed | secondary metabolism.isoprenoids.mevalonate pathway.acetyl-CoA C-acyltransferase | 1.438 |
| loc_os02g08100 | 4CL1 (4- AMP-binding domain containing protein, expressed | secondary metabolism.phenylpropanoids.lignin biosynthesis.4CL | 1.205 |
| loc_os08g38900 | caffeoyl-CoA O-methyltransferase, putative, expressed | secondary metabolism.phenylpropanoids.lignin biosynthesis.CCoAOMT | 1.192 |
| loc_os11g40690 | dehydrogenase, putative, expressed | secondary metabolism.phenylpropanoids.lignin biosynthesis.CAD | 1.012 |
| loc_os02g09490 | ATCAD5 dehydrogenase, putative, expressed | secondary metabolism.phenylpropanoids.lignin biosynthesis.CAD | 1.099 |
| loc_os02g43830 | 3-isopropylmalate dehydratase small subunit 2, putative, expressed | secondary metabolism.sulfur-containing.glucosinolates.synthesis.aliphatic.methylthioalkylmalate isomerase small subunit (MAM-IS) | 1.827 |
| | | **Hormone Metabolism** | |
| loc_os07g18120 | aldehyde oxidase, putative, expressed | hormone metabolism.abscisic acid.synthesis-degradation | 1.392 |
| loc_os08g35740 | 12-oxophytodienoate reductase, putative, expressed | hormone metabolism.jasmonate.synthesis-degradation.12-Oxo-PDA-reductase | 1.215 |
| | | **Co-factor and vitamine metabolism** | |
| loc_os06g34040 | DJ-1 family protein, putative, expressed | Co-factor and vitamine metabolism.thiamine | 1.995 |
| | | **Tetrapyrrole synthesis** | |
| loc_os08g41990 | aminotransferase, putative, expressed | tetrapyrrole synthesis.GSA | 1.049 |
| | | **stress** | |
| loc_os07g01660 | dirigent, putative, expressed | stress.biotic.PR-proteins | 1.412 |
| | | **Redox** | |
| loc_os02g56850 | glutathione reductase, putative, expressed | redox.ascorbate and glutathione.glutathione | 1.038 |
| loc_os04g46960 | glutathione peroxidase domain containing protein, expressed | redox.ascorbate and glutathione.glutathione | 1.222 |
| | | **Nucleotide metabolism** | |
| loc_os03g61600 | phosphoribosylformylglycinamidine synthase, putative, expressed | nucleotide metabolism.synthesis.purine.AIR synthase | 1.046 |
| | | **Biodegradation of Xenobiotics** | |
| loc_os03g21460 | metallo-beta-lactamase family protein, putative, expressed | Biodegradation of Xenobiotics.hydroxyacylglutathione hydrolase | 1.003 |
| | | C1-metabolism | |
| loc_os09g27420 | formate--tetrahydrofolate ligase, putative, expressed | C1-metabolism.formate-tetrahydrofolate ligase | 1.053 |
| | | **Miscellaneous** | |
| loc_os01g71350 | glycosyl hydrolases family 17, putative, expressed | misc.beta 1,3 glucan hydrolases.glucan endo-1,3-beta-glucosidase | 2.654 |
| loc_os11g20160 | O-methyltransferase, putative, expressed | misc.O-methyl transferases - lateral root | 8.719 |
| loc_os08g06100 | O-methyltransferase, putative, expressed | misc.O-methyl transferases | 1.976 |
| loc_os03g13540 | Ser/Thr protein phosphatase family protein, putative, expressed | misc.acid and other phosphatases | 1.002 |

| | | | |
|---|---|---|---|
| loc_os03g01300 | LTPL114 - Protease inhibitor/seed storage/LTP family protein precursor, expressed | misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein | 1.858 |
| loc_os04g46810.1 | LTPL120 - Protease inhibitor/seed storage/LTP family protein precursor, expressed | misc.protease inhibitor/seed storage/lipid transfer protein (LTP) family protein | 1.184 |
| | | **Protein Synthesis** | |
| loc_os02g52880 | ybaK/prolyl-tRNA synthetase family protein, putative, expressed | protein.aa activation.proline-tRNA ligase | 1.06 |
| loc_os11g29190 | 40S ribosomal protein S5, putative, expressed | protein.synthesis.ribosomal protein.eukaryotic.40S subunit.S5 | 1.003 |
| loc_os12g38000 | 60S ribosomal protein L8, putative, expressed | protein.synthesis.ribosomal protein.eukaryotic.60S subunit.L8 | 2.359 |
| loc_os07g14280 | WD domain, G-beta repeat domain containing protein, expressed | protein.targeting.secretory pathway.plasma membrane | 1.247 |
| | | **Signalling** | |
| loc_os01g63800 | pleckstrin homology domain-containing protein, putative, expressed | signalling.phosphinositides | 1.105 |
| loc_os08g33370 | 14-3-3 protein, putative, expressed | signalling.14-3-3 proteins | 1.419 |
| loc_os08g37490 | 14-3-3 protein, putative, expressed | signalling.14-3-3 proteins | 1.134 |
| | | **Development/storage** | |
| loc_os05g02520 | cupin domain containing protein, expressed | development.storage proteins | 1.302 |
| | | **Transport** | |
| loc_os02g57240 | oxidoreductase, aldo/keto reductase family protein, putative, expressed | transport.potassium | 2.31 |
| loc_os02g44080 | aquaporin protein, putative, expressed | transport.Major Intrinsic Proteins.TIP | 1.348 |

**Suppl Table 3. The sequences of gene specific forward and reverse primers used for the qRT-PCR analysis**

| Gene Name | Accession number | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|---|---|---|---|
| OsCyclophilin 2 | LOC_Os02g02890 | GTGGTGTTAGTCTTTTTATGAGTTCGT | ACCAAACCATGGGCGATCT |
| OsAOC | LOC_Os03g32314 | TGCCTCAACAACTTCACCAACTA | CACATGCCGCAATTAACACTAAA |
| OsOPR7 | LOC_Os08g35740 | CTCAACCACCGGTTTCCTCA | TCCATGCATCAGTCTGCTCT |
| OsPAL1 | LOC_Os02g41630 | AAGGTGTTCCTCGGCATCAG | GGCAATGGCGATGGGATCTT |
| Os4CL3 | LOC_Os02g08100 | CTCACCCGGAGATCAAGGAC | CCTCGGTGATTTCTGAGCCT |
| OsCOMT | LOC_Os08g38900 | TCATCACGGACAAGCACCAG | GACACCCACCTCGATTGTCC |