Adaptation to salt stress in rice –

How jasmonates contribute to the response to high salinity

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Karlsruhe, im Juni 2014

Mohamed Hazman

To My Father and My Mother

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Mohamed Hazman

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Abbreviations

ABA: Abscisic acid

APX: Ascorbate peroxidise

CAT: Catalase

cpm2: Coleoptile photomorphogenesis 2

DAF-2DA: 4,5-diaminofluorescein diacetate

DPPH: 2,2-dipheny-1-picrylhydrazyl

GR: Glutathion Reductase

GST: Glutathion-s-transferase

H_2O_2: hydrogen peroxide

JA: Jasmonic acid

JA-Ile: Jasmonate Isoleucine

JAZ: Jasmonate ZIM domain

MDA: Malondialdehyde

NaCl: Sodium chloride

NCED: 9-cis-epoxycarotenoids dioxygenase

NHX: Na+/H+ Antiporters

NO: Nitric Oxide

NR: Nitrate Reductase.

$O2^{\cdot -}$: superoxide anion

$O2^{\cdot 1}$: Singlet oxygen

$OH^{\cdot}$: superoxide anion

OPDA: Oxi-phytodecatrenoic acid

OXO: Oxalic acid oxidase

POD: peroxidises

ROS: Reactive Oxygen Species

SAT: Serine acetyltransferase

SOD: Superoxide Dismutase

TFC: Total Flavonoids content

TPC: Total phenolics content

Zusammenfassung

Bodenversalzung ist ein wichtiger Umweltfaktor, der pflanzliches Wachstum und Produktivität in der Landwirtschaft einschränkt. Aufgrund ihrer sessilen Lebensweise müssen Pflanzen ihr Wachstum und ihre Entwicklung durch Integration verschiedener Hormonsignale an die Standortbedingungen anpassen. Das Oxylipin Jasmonsäure und seine Metabolite, insgesamt als Jasmonate bezeichnet, vermitteln Reaktionen auf biotischen und abiotischen Stress. Ihre Funktion für die Reaktion auf Salzstress ist jedoch bisher nicht vollständig aufgeklärt worden. Daher wurde untersucht wie zwei Jasmonatbiosynthesemutanten (cpm2 und hebiba) im Vergleich mit dem Wildtypen auf Salzstress reagieren.

Diese Genotypen wurden hinsichtlich Morphologie, Physiologie und molekularbiologischer Antworten verglichen. Überraschenderweise legte der Phänotyp der Jasmonatbiosynthesemutanten nahe, dass sie weniger empfindlich für Salzstress sind, was sich an weniger Stresssymptomen an zweiten und dritten Blättern sowie längeren Wurzeln von Keimlingen, die bei hohen Salzgehalten wuchsen, zeigte. Interessanterweise haben die beiden Mutanten cpm2 und hebiba weniger Na^+-Ionen in Blättern angesammelt. Komponenten, die Schädigung durch oxidativen Stress anzeigen (MDA und H_2O_2), waren in Wildtypblättern in größerem Maß vorhanden. Jedoch wurde lösliches Prolin, das als Antioxidans und kompatibler Solut diskutiert wird, in den Mutanten in geringeren Mengen nachgewiesen. Weiterhin wurde beobachtet, dass Rohextrakte der Mutante über eine höhere Kapazität zur Detoxifizierung von in vitro erzeugten reaktiven Sauerstoffspezies (ROS) verfügten, als Rohextrakte des Wildtyps, was auf eine höheres antioxidatives Potential der Mutantenextrakte hindeutet. Profile der Aktivität antioxidativer Enzyme zeigten, dass Superoxiddismutase (SOD) und Peroxidase (POD), Glutathione Reduktase (GR) und Glutathione-S-Transferase (GST) im Spross beider Mutanten im Vergleich zum Wildtyp eine höhere Aktivität aufwiesen. Genexpressionsanalysen von ausgewählten Genen in der Salzstress-induzierten Signalleitung zeigte, dass in den Mutanten das Transkript eines Na^+/H^+-Antiporters in Vakuolen (OsNHX1) weniger stark induziert wird als im Wildtyp, was nahe legt, dass die Vakuolen weniger Na^+ aufnehmen. Die Rolle von Abscisinsäure (ABA) wurde über die

Änderung in der Transkriptmenge des ABA-Biosyntheseschlüsselenzyms OsNCED5 (9-cis-Epoxycarotenoiddioxygenase) untersucht, das in Mutanten stärker induzierbar war als im Wildtyp. Jedoch war der Gehalt an ABA in Wildtyp und Jasmonatbiosynthesemutanten vergleichbar, was darauf hinweist, dass nicht die Biosynthese sondern die Empfindlichkeit der Mutanten für ABA verändert sein könnte. Zudem wurde festgestellt, dass die Expression des Gens OsNR, welches für das Schlüsselenzym der Biosynthese von Stickstoffmonoxid (Nitratreduktase) kodiert, durch Salzstress in Mutanten stärker induziert wurde und dass in Schließzellen von Mutanten mehr Stickstoffmonoxid akkumuliert als in denen des Wildtyps. Die endogenen Gehalte von Jasmonasäure, Jasmonsäure-Isoleucin und vor allem OPDA waren im Wildtyp, nicht jedoch in den Mutanten, erhöht. Basierend auf diesen Ergebnissen schlagen wir vor, dass Jasmonate die Biosynthese von Stickstoffmonoxid beeinflussen und somit das Schliessen von Spaltöffnungen verändern könnte. Aufgrund einer veränderten Stomataöffnung könnte sich die Transpirationsrate in den Mutanten verlangsamen , was zu einem geringen Wurzel-Spross-Transport von Natriumionen führen würde. Da dann weniger Natrium von den Wurzeln in die Blätter gelangt, leiden Jasmonasäurebiosynthesemutanten weniger unter oxidativem Stress, da das System zur Detoxifizierung von ROS weniger stark durch Na^+-Ionen beschädigt wird.

Abstract

Salinity is a major environmental factor limiting plant growth and productivity in agriculture. Due to its sessile lifestyle, plants must adjust their growth and development under such conditions through the integration of hormone signaling. The oxylipin jasmonic acid and its metabolites, collectively known as jasmonates are important plant signalling molecules that mediate responses to biotic and abiotic stresses; nevertheless, its role under salt stress is not completely uncovered. The response of two jasmonate biosynthesis rice mutants (cpm2 and hebiba) to salt stress was investigated in comparison to their wild type.

These genotypes were compared on the level of morphology, physiology and molecular biology. Surprisingly, the phenotype of jasmonate biosynthesis mutants suggested that they are less sensitive to salinity, illustrated by less stress damage symptoms in second and third leaves, and longer roots under salt stress in seedlings exposed to high salt concentrations. Interestingly, both cpm2 and hebiba plants accumulated smaller amounts of Na^+ ions in their leaves. Oxidative damage parameters (MDA and H_2O_2) were higher in wild type leaves. Nevertheless, soluble proline, discussed as an antioxidant and osmoprotectant, was less abundant in the mutants. Furthermore, it was observed that the crude extract of the mutants detoxified in vitro produced reactive oxygen species (ROS) more efficiently than wild type extracts reflecting a higher antioxidative power. The profile of antioxidant enzyme activities showed that Superoxide dismutase (SOD) and Peroxidases (POD), Glutathione Reductase (GR) and Glutathion-s-transferase (GST) performed better in the shoots of both mutants compared to the wild type. Gene expression analysis of selected genes in the signaling pathway of salinity revealed that the mutants showed significantly lower inducibility of the vacuolar Na^+/H^+ antiporter encoding gene (OsNHX1) suggesting less Na^+ uptake into vacuoles. The role of abscisic acid (ABA) was investigated through measuring the expression of one of the ABA key biosynthesis enzymes OsNCED5 (9-cis-epoxycarotenoid dioxygenase) which was found to be higher in the mutants comparing to WT, nevertheless, the endogenous level of ABA was comparable in both wild type and jasmonate biosynthesis mutants, indicating that not the biosynthesis but the sensitivity

for ABA in the mutants might be altered. Furthermore it was found that OsNR gene expression (encoding for nitrate reductase, a key enzymes in NO production) was induced by salinity more strongly in the mutants, and the stomatal guard cells of the mutants accumulate more NO than that of the wild type. The endogenous levels of JA, JA-Ile and especially OPDA were elevated in response to salt stress in case of wild type only but not changed in the mutants. Based on these results, we suggest that jasmonates may affect the biosynthesis of NO and in this way alters stomata closure. Due to altered stomatal opening, the transpiration stream might be slower in the mutants leading to less root-to-shoot sodium ions transfer, As a result of transferring less sodium from roots to its leaves, JA biosynthesis mutants suffer less from oxidative stress as its ability to scavenge ROS was not totally damaged by Na^+ ions.

1- Introduction

Plants are the backbone of not only our life, rather than all life on earth, it is an essential resource of human wellbeing. Everything we eat comes directly or indirectly from plants. Throughout human history, approximately 7000 different plant species have been used as food. 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 every day, additionally it is a model species for monocotyledonous and cereal plants (Cotsaftis and Guiderdonis, 2005). Due to the lacking of sessile way of living (unlike animals), plants evolved many unique and sophisticated physiological mechanisms in order to cope with the unfavourable environmental changes, which come from natural reasons or from human activities. Stress usually, defined as the external factor that exerts disadvantageous influence on the plant. In most cases, stress is measured in relation to plant survival, crop yield, growth (biomass accumulation), or the primary assimilation process (CO_2 and minerals uptake), which are related to overgrowth (Taiz and Zeiger, 2002). Saline (salt-affected) soil is one of the major serious problems that limit the securing of food reserves for humans needs through agriculture.

1.1 What is salinity and why is it a tough enemy to the agriculture?

Salinity is *defined* as the presence of excessive amount of soluble salt that hinder or affect negatively the normal function needs for plant growth. It is measured in terms of electroconductivity (ECe), or exchangeable Na^+ percentage (ESP) or with the Na^+ absorption ratio (SAR) and pH of the saturated soil past extract. Therefore saline soil are those with ECe more than 4 dSm^{-1} equvilant to 40 mM NaCl, ESP less than 15% and pH below 8.5 (Waisel, 1972 and Abrol, 1998). Table 1 showing the three different level of salinity and its relations to the value of ECe as a common factor for salinity degree classifications.

Approximately 20% of the irrigated lands in the world are presumably affected by soil salinization (yeo,1999). Saline lands are not only distributed in desert and semi-desert regions but also frequently occurs in fertile alluvial plains, rivers, valleys, and coastal regions, close to densely, populated areas and irrigation systems. For example, the

agricultural Egyptian economy suffers from severe salinity problems 33% of the cultivated lands are already salinized (Mohammed *et al*, 2007).

Degree of salinity	Site characteristics	Use	Species
Low salinity (EC_e^*=2 to 4 dSm^{-1})	Natural salinity; often seasonally dry Irrigation salinity; can be waterlogged after irrigation	Cropping	Low-moderate salt tolerance
Moderate salinity (EC_e= 4 to 8 dSm^{-1})	Dryland salinity; often seasonally waterlogged	Crop-pasture rotations	Moderate-high salt tolerance
High salinity (EC_e > 8 dSm^{-1})	Discharge areas; can be seeping or dry according to season	Grazing or revegetation	Halophytes

Table 1. Classes of soil salinity and expected land use.

(*EC_e of a saturated extract which approximates saturated field water content).

source: FAO Land and Plant Nutrition Management Service

The costs of salinity to agriculture are estimated conservatively to be about $US 12 billion a year and it is expected to increase as soil are further affected (Gnassemi *et al*, 1995). Salinity also could be severely destructive to the agricultural economy as a result of natural causes. For instance, recent deposition of toxic salt sediments and sea intrusion in tsunami-affected areas of Maldives damage >70% of agriculture land, destroyed >370,000 fruit tree and affected around 15,000 farmers, with cost estimated at around AU$ 6.5 million (FAO, 2005). Most of grain crops and vegetables are glycophytes (salt-sensetive flora), therefore are highly susceptible to soil salinity even when the soil ECe is < 4dSm-1. Table 2 shows how most of our essentials food crops are susceptible to salinity stress. Different threshold tolerance ECe and different rate of reduction in yield beyond threshold tolerance indicates variations in salt tolerance among those crops (Chinnusamy *et al*, 2005).

Crop	Threeshold salinity	Decrease in yield
	dSm^{-1}	Slope % per dSm^{-1}
Bean (*Phaseolus vulgaris* L.)	1.0	19.0
Eggplant (*Solanum melongena* L.)	1.1	6.9
Onion (*Allium cepa* L.)	1.2	16.0
Pepper (*Capsicum annuum* L.)	1.5	14.0
Corn (*Zea mays* L.)	1.7	12.0
Sugarcane (*Saccharum officinarum* L.)	1.7	5.9
Potato (*Solanum tuberosum* L.)	1.7	12.0
Cabbage (*Brassica oleracea* var. *capitata* L.)	1.8	9.7
Tomato (*Lycopersicon esculentum* Mill.)	2.5	9.9
Rice, paddy (*Oryza sativa* L.)	3.0	12.0
Peanut (*Arachis hypogaea* L.)	3.2	29.0
Soybean [*Glycine max* (L.) Merr.]	5.0	20.0
Wheat (*Triticum aestivum* L.)	6.0	7.1
Sugar beet (*Beta vulgaris* L.)	7.0	5.9
Cotton (*Gossypium hirsutum* L.)	7.7	5.2
Barley (*Hordeum vulgare* L.)	8.0	5.0

Table 2. Many important crops are susceptible to soil salinity† (Maas, 1990).Crop Threshold salinity Decrease in yielddS m^{-1} Slope % per dS m^{-1}.

1.2 Types and Causes of soil salinization

We are living on a salty planet. 97.5 % of all water on earth is ‘salt water’, leaving only 2.5% to be as fresh water. Only lower than 0.1% of this fresh water (~0.007% of all water on earth) is accessible for direct human use.

Salt-affected lands occur in practically all climate regions, from humid tropics to the Polar Regions. Saline soil can be found at different altitudes, from below sea levels (e.g. around the Dead Sea) to mountains rising above 5000 meters, such as Rocky Mountains (Singh at Chatrath, 2001).

There are two types of salinity depending on the source and the way of salinization, the primary (natural salinity) and secondary (human-induced) salinity. The primary or natural salinity resulted from the accumulation of salts over long period of time, through two natural processes, in the soil or in the ground water. The first process is the weathering of parent materials containing soluble salts. Weathering process breakdown rocks and release soluble salts, mainly sodium chloride. In the second process oceanic salt carried inland by wind and deposited by rain fall, and most of salt is sodium chloride.

Secondary or human-induced salinity resulted from human activities that change the hydrologic balance of the soil between water applied (irrigation or rainfall) and water used by crops (transpiration). The most common causes are (i) land clearing and the replacement of perennial vegetation with annual crops, and (ii) irrigation schemes using salt-rich irrigation water or having insufficient drainage (Munns, 2002).

In some case, the canal low salt fresh water is not enough for the agricultural needs in northern part of Egypt, mainly the delta where rice planting is extensively applied. Therefore the farmers were obligated to use the drainage water to irrigate their fields. In case of using fresh canal water, the salinity in the top soil (0-60 cm) decreased by 3-12%, while it increased to be between 21-26% in case of using drainage water (Abdelmawgod, 2005). Table 3 shows the area of the irrigated land and the percentage ratio of the salt-affected soil in many agricultural countries in different positions in the world.

Country	Total land area cropped Mha	Area irrigated		Area of irrigated land that is salt-affected	
		Mha	%	Mha	%
China	97	45	46	6.7	15
India	169	42	25	7.0	17
Soviet Union	233	21	9	3.7	18
United States	190	18	10	4.2	23
Pakistan	21	16	78	4.2	26
Iran	15	6	39	1.7	30
Thailand	20	4	20	0.4	10
Egypt	3	3	100	0.9	33
Australia	47	2	4	0.2	9
Argentina	36	2	5	0.6	34
South Africa	13	1	9	0.1	9
Subtotal	843	159	19	29.6	20
World	1,474	227	15	45.4	20

Table 3: Global estimate of secondary salinisation in the world's irrigated lands. Source: Ghassemi *et al*. (1995)

1.3 Types of plants based on general salt tolerance

Based on the response to high concentration of salts, plants can be divided into two broad groups, halophytes (salt-tolerant) and glycophytes (not salt tolerant). Halophyte is a group of plants that is able to grow even in high saline conditions. Halophytes commonly require some salt (soil solution c. 10–50 mM NaCl) to reach maximum growth, and a few halophytes, for example Atriplex nummularia (old man saltbush), grow best around 100 mM NaCl. Many halophytes can grow in full strength or even

concentrated seawater (mangroves) where the molarities of NaCl almost close to 500mM (Atwell *et al*, 1999).

Glycophytes or (sweet plants) are not salt tolerant, most of cultivated crops by humans belong to this group, as rice, wheat and maize. As an interesting example, Porteresia coarctate or wild rice, the halophyte relative to the cultivated rice. The wild rice is more efficient in the protection of its photosynthesis machinery against free radical produced by salinity stress when exposed to salinity of 400mM NaCl (Bose *et al*, 2013).

1.4 Effect of salinity on plants

Salinity is a single word; nevertheless its effect on plants and plants reaction toward it needs pages to be discussed, general points summarized in Figure 1. The salinity stress triggered initially two main harmful effects, namely, i) osmotic (reduced water uptake) and ii) specific ion toxicity stress (mainly Na^+ ad Cl^-), which leads to the third generative stress – iii) oxidative stress- where uncontrolled production of ROS (Reactive Oxygen Species) as superoxide radicals ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyle radicals ($OH^{\cdot}$). Those unstable molecules accumulated to toxic levels and trigger an oxidative damage effect on the valuable biochemical molecules as proteins, enzymes, DNA and RNA. (Pessarakli, 2001 and Sharma, *et al*., 2012).

On the level of morphology, the plants under salinity stress showing many external symptoms as in Figure 2. The tip of the affected leaves turn white, chlorotic patches appear on some leaves and plant stunting and reduced tillering. Growth inhibition is the primary injury that leads to other symptoms although programmed cell death may also occur under severe salinity shock. (IRRI site)

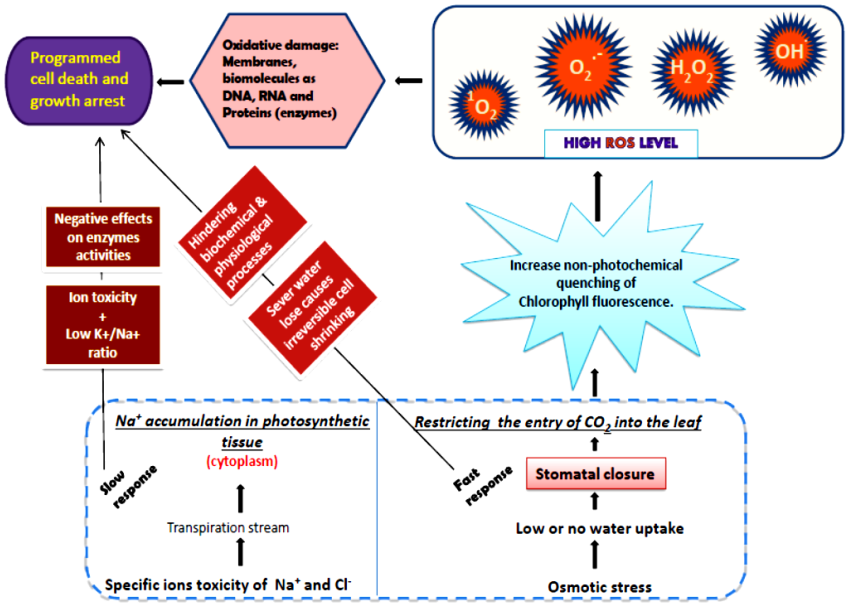

Figure 1: General physiological effects triggered by salinity stress on plants growth.

Figure2: The symptoms of salinity stress on the vegetative growth of rice seedling in the field, whitening and browning of leaves (source: IRRI).

1.4.1 The impact of salinity stress on the water status of plants

In fact, in saline soils, although water is present it is unavailable to plants because it retained by the ions in the soil, such as Na^+ and Cl^-. Under non-stress conditions, intracellular osmotic potentials (Ψ) is generally more negative than that of the soil solution, resulting in water influx into roots according to water potential gradient.

Because of dissolved ions that decrease extracellular Ψ_{osm}, salinity stress immediately reduce $\Delta\Psi$ thus water influx. If the water potential gradient is reversed due to sever salinity / osmotic stress, water efflux from roots (dehydration) can occur (Horie *et al*, 2012).

Typically, as the water content of the plant decreased, its cell shrink and the cell wall relax. This decrease in cell volume results in lower turgid pressure, additionally the plasma membrane become thicker and more compressed because it covers a smaller area than before. Removal of water from the membrane disturbs the normal bi-layer structure and results in the membrane becoming exceptionally porous when desiccated (Taiz and Zeiger, 2002).

Stress within the lipid bi-layer may also results in displacement of membrane proteins and this contributes to loss of membrane integrity, selectivity, disruption of cellular compartmentalization and a loss of enzyme activity, which are primarily membrane based. In addition to membrane damage, cytosolic and organelle proteins may exhibit reduction activity or may even undergo complete denaturation when severely dehydrated. The high concentration of cellular electrolytes due to the dehydration of protoplasm may also cause disruption of cellular metabolism (Mahajan and Tuteja, 2005).

1.4.2 Sodium Ion-specific stresses

1.4.2.1 Sodium entry to the plant cell

Sodium toxicity is one of the most formidable challenges for crop production world-wide. Nevertheless, despite decades of intensive research, the pathways of Na+ entry into the roots of plants under high salinity are not definitively known (Kronzucker and Britto, 2010).

In general, uptake of ions from the external environment through the cell wall of root cells is a passive process driven by diffusion or mass flow (Marscher, 1995). The primary cell wall consists of cross-linked cellulose, hemicelluloses and glycoproteins with build pores with maximum diameters of 5 nm (Carpita *et al*. 1979). Since hydrated

ions are only upto 20% of the pore size, here no restriction of the movement of ions should occur.

Under typical physiological conditions, plants maintains a high potassium/sodium (Na^+/K^+) ratio in their cytsol with relatively high K^+ (100-200mM) and low Na+ concentration (1-10mM). Given the negative electrical membrane potential difference at the plasma membrane (-140mV). A rise in extracellular Na+ concentration will establish large sodium electrochemical potential gradient that will favor the passive transport of sodium from the environment into the cytosol (Blumwald *et al*, 2000).

Excess extracellular Na+ is gained the ability to inter the cells through its plasma membrane using the high affinity K^+ transporters (HKT) family members in different tendencies and non-selective cation channels (NSCCs). Those transporters are used in the up taking of the potassium ions (80%) which are very essential micronutrients for the plant as K^+ ions are needed to adjust turgor pressure and stomata movement, and also essential as cofactors for many metabolic enzymes. Under high salinity stress, the negatively charged plasma membrane HKT channels cannot distinguish or select potassium ions from sodium; hence Na^+ ions used KHT channels excessively to inter the cytoplasm (Szczerba *et al*., 2008).

1.4.2.2 Sodium toxicity

The sodium specific ion toxicity mechanisms within the cell are very poorly understood unlike the strictly osmotic effects. One reason for that is, there is little certainly regarding intracellular Na^+ ions concentration, particularly in the cytosol as its vary dramatically from one another depending on the method used even within the same organism, while in case of cytosolic level of K^+, there is a strong consensus from many independent reported, that it is around 100mM. Additionally, the maintenance of a high K^+/Na^+ ratio in the cytosol of plant root cells is becoming very clear and frequently described in the literature as being a critical determinant under salinity stress (Kronzucker *et al*, 2013).

The cytoplasm of eukaryotic cells has evolved to work best within a limited range of concentrations of solutes, and particularly of certain ions. Excluding these ranges for inorganic (and some organics) ions (including potassium) creates problems of

macromolecular structure and hence enzyme activities and nucleic acid metabolism (Wyn Jones *et al*, 1979).

The first target for sodium ion toxicity is potassium ion homeostasis. K^+ homeostasis is critical for proper plant cell function, so the question that is strongly applied, whether its disruption by Na^+ may be sufficient to explain a long part of Na^+ toxicity. Na+ has been shown to suppress K^+ influx in both high- and low – affinity ranges, particularly at mill molar concentration. In most cases, potassium transport inhibition is believed to be mediated my Na^+ binding to the outside of carrier and channels (Szczerba *et al*, 2008).

The activity of most enzymes is negatively affected by high salt concentrations due to perturbation of the hydrophobic–electrostatic balance between the forces maintaining protein structure. However, toxic effects on cells occur even at moderate salt concentrations of about 100 mM, unveiling specific salt toxicity targets (Serrano 1999).

1.5 The sensing of salinity stress: pain can save life

The plant cannot launch a well planned adaptation mechanism without starting well organized signal transduction pathway. Nevertheless, in order to trigger any pathway of signal transduction, firstly the stress must be properly sensed. Briefly, we will try to have a look on what we really know about the initial or primary sensing, as it is the corner stone of a successful adaptation mechanism on the levels of molecular biology, biochemistry and physiology.

1.5.1 Primary sensing of salinity stress

1.5.1.1 Primary sensing of water deficient

Sensing water deficient or dehydration is not possible to be studied from the theories of traditional signal perception. The normal models telling us about a legend bind to a receptor located on the external side of the plasma membrane, but in case of osmotic stress, there is no chemical to be perceived. Dehydration causes a set of changes in the cell as solute (organic and inorganic) concentration, cell volume, plasma membrane integrity and proteins degradation. One or a group of those effects may be perceived from the cell stress signals.

As previously mentioned, deficient in the water content of the soil environment might be sensed as increase in the salt concentration around root surface and / or increase in the osmotic pressure of the root cells. Although it is easy enough to imagine that higher plants are sensing osmotic stress, there are no soiled evidences about the identification of water sensors or potential low water sensors. (Yakota *et al*, 2006).

Some information on the sensing of osmotic stress is available from bacteria, and for some eukarya including yeast. Nevertheless, it is still not completely clear, even in these organisms, how osmotic stress is sensed (Hohmann, 2002). There are some lines of evidences that plants also show some promising similar identities.

1.5.1.2 Histidine kinases (HKs): just one example

KHs are a well studied group of sensor molecules which undoubtedly sensing environmental changes including osmotic stress. The histidyl-aspartyl (His-Asp) phosphorelay system, also known as two component regulatory systems and which are involved in cytokinin signal transduction have been studied extensively in bacteria, arabidopsis and maize, which use these systems to sense the respond to divers of environmental stimuli (Yonekura-Sakakibara *et al*, 2004)

The signaling cascade typically consists of three functional modules: a sensory histidine kinase (HK), a histidine phosphotransfer protein (HP), and a response regulator (RR). In these systems, signals are transmitted by a phosphoryl group transfer between His and Asp residues. (Mizuno, 1998).

Wang *et al*, 2012 reported about the identification of a novel histidine kinase, zmHK9, which mediate drought tolerance through the regulation of stomatal movement after its overexpression in arabidopsis. In the ZmHK9 overexpressing plants, the stomatal complex size and stomatal density were much lower than the wild type. Wohlbach *et al*, 2008 suggested that AtHK1 is a major and important osmosensor and has a connection to ABA synthesis, while Kumar *et al*, 2012 presented relatively contradictory conclusions.

1.5.1.2 Initial sensing of sodium ion-stresses

In order to cope with the high salinity stress, it is necessary for the plants, especially glycophytes, to reduce the accumulated sodium ions in its photosynthetic tissue. However, the sodium ion has to be sensed, and then the plant cells start transductions of the sensed stress. Currently, it is not certainly confirmed whether extracellular or intracellular sodium ions are sensed and how, and in both cases the exact solid evidence for a sodium ion molecular receptor is absent (Zhu, 2007).

Even though the molecular identity of Na+ sensors remains elusive, the plasma membrane Na+/H+ antiporter SOS1 (Salt Overlay Sensitive 1) is a probable candidate (Shi *et al*, 2000). The transport activity of SOS1 is essential for sodium efflux from Arabidopsis cells, but additionally, its long cytoplasmic tail is thought to be involved in Na+ sensing (Conde *et al*, 2011).

1.5.2 Second intracellular signaling and salt adaptation mechanisms

As long as the plant cell was able properly to recognize the salinity stress initial signals with its two main effects, dehydration and ion toxicity, so the signal transduction pathways will be activated. The molecular trafficking of the stressed cell will be significantly changed, mainly gene expression (ex: transcription factors and stress-related genes) and metabolic readjustment (phytohormones, reactive oxygen species and osmoprotectants), leading to altering the physiological state (ex: stomatal closure) that result in better adaptation towards salinity or any kind of unfavorable conditions.

During the next titles, we would like to collect the main big lines with some interesting details of the salt stress adaptation mechanisms story. The second intracellular signaling or signal transduction pathway for each mechanism should be not being introduced separately.

1.5.2.1. Adaptive response to water deficient stress

1.5.2.1.1 Immediate closure of stomata

Stomata form pores on leaf surface that regulate the uptake of carbone dioxide (CO_2) for photosynthesis and the loss of water vapour during transpiration (Ng *et al*, 2001).

Plants' leaves close their stomata immediately, the process completed in few minutes, on sensing an increase in the leaf-air vapour pressure difference, even if the roots have sufficient water (Mott and Parthurst, 1991). Salinity/osmotic stress directly or indirectly via hormonal regulation induce a stomatal closure, which leads to a reduction in the evaporation and overall water transport (Horie *et al*, 2012).

1.5.2.1.1.1 Role of Abscisic acid (ABA)

Abscisic acid (ABA) is a plant stress hormone and one of the foremost important signalling molecules in plants, which plays versatile functions in regulating many developmental processes and adaptive responses (Santer *et al*, 2009 and Culter *et al*, 2010). Water deficit induces expression of the gene encoding NCED (9-cis-epoxycarotenoid dioxygenase), which catalyzes a critical committed step in drought-induced ABA biosynthesis (Iuchi *et al*. 2000; Xiong and Zhu 2003). Water deficit induces, by transcriptional and post-transcriptional regulation, activity of the BG1 β-glucosidase, which specifically cleaves biologically inactive ABA-glucose ester (ABA-GE) to form ABA (Lee *et al*. 2006). Further, water deficit causes acidification of the leaf symplast and alkalinzation of the apoplast—both mechanisms enhancing accumulation of ABA in the leaf apoplast—leading to increased ABA arriving at the guard cells to initiate closure in response to water deficit (Wilkinson and Davies 1997 and Christmann *et al*. 2004).

As in Figure one by (McAinsh *et al*., 1990), ABA triggers cytosolic calcium ($[Ca^{2+}]_{cyt}$) increases $[Ca^{2+}]_{cyt}$ elevations activate two different types of Cl^- anion channels: Slow-activating sustained (S-type) (Schroeder and Hagiwara, 1989) and rapid transient (R-type) Hedrich *et al*., 1990) anion channels. Both mediate Cl^- anion release from guard cells, causing depolarization. This change in membrane potential deactivates inward-rectifying K^+ (K^+_{in}) channels and activates outward-rectifying K^+ (K^+_{out}) channels (Schroeder *et al*., 1987), resulting in K^+ efflux from guard cells (from tonoplas to cytoplasm then to the apoplastic extracellular space). In addition, ABA causes an alkalization of the guard cell cytosol (Blatt and Armstrong, 1993) which directly enhances K^+_{out} channel activity (Ilan *et al*., 1994; Miedema and Assmann, 1996) and down-regulates the transient R-type anion channels (Schulz-Lessdorf *et al*., 1996). The

sustained efflux of both anions and K^+ from guard cells via anion and K^+_{out} channels contributes to loss of guard cell turgor, This leads to water loss then loss of turgor, which leads to stomatal closing (Pandey *et al*. 2007).

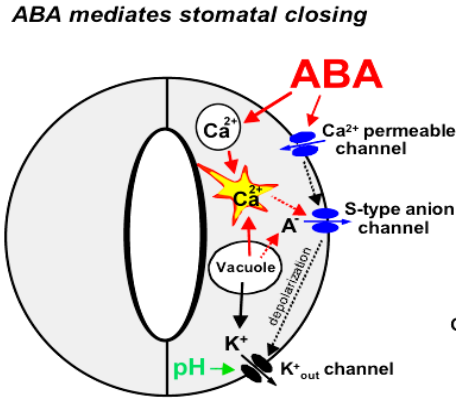

Figure 3. A model for roles of ion channels in ABA signaling. Source: McAinsh *et al*, 1990.

1.5.2.1.1.2 NO (Nitric Oxide) contribution

Nitric oxide (NO) has now gained significant place in plant science, mainly due to its properties (free radical, small size, no charge, short-lived, and highly diffusible across biological membranes) and multifunctional roles in plant growth, development, and regulation of remarkable spectrum of plant cellular mechanisms (Siddiqui *et al*, 2011). Stomatal closure, initiated by ABA, is affected through a complex symphony of intracellular signaling in which NO appears to be one component. This conclusion is being to be strongly believed cause there are many independent observations supporting it, as exogenous NO induces stomatal closure, ABA triggers NO generation, removal of NO by scavengers inhibits stomatal closure in response to ABA, and ABA induced stomatal closure is reduced inmutants that are impaired in NO generation. Stomatal closure, initiated by ABA is affected through a complex symphony of intracellular signaling in which NO appears to be one component (Neill *et al*, 2000).

In spite the role of NO in stomatal closure under osmotic stress condition is not a clear absolute, its involvement in the well know adaptive response (stomatal closure) cannot be excluded. Drought promoted the production of NO in pea and tobacco (Leshem and Haramaty 1996; Gould *et al*. 2003). While Tian and Lei, 2006, reported that the NO donor, sodium nitroproside (SNP) enhanced wheat seedling growth, kept high relative

water content and increased the alleviated the injury of oxidative. Recently, Lamattina and Garcı´a-Mata, 2014, reported that under osmotic stress the NO donor, sodium nitroproside (SNP), clearly enhanced the adaptive responses of wheat plants through the induction of stomatal closure.

Uniformly, if the extracellular hero of the stomatal closing story was ABA, Ca^{++} ion is the main intracellular player. The effect of ABA and NO could meet in the issue Ca^{++} signaling manipulation. In animals, NO has been reported to increase intracellular $[Ca^{++}]_i$ concentrations both in a cGMP (cyclic guanosine monophosphate) dependent or cGMP-independent way. These $[Ca^{++}]_I$ were reported to be a consequence of uptake form the extracellular space or due to Ca++ liberation from intracellular stores (Berkerls *et al*, 2000). In plants, some evidences show that NO increased cGMP levels which in turn stimulate the expression of plant defense genes. cGMP has a main role as second messenger molecules in the activation of non-selective Ca++ permeable cation channels in the guard cells of Arabidopsis (Wang *et al*, 2009).

1.5.2.1.2. Decreasing leaf area

Because turgor pressure reduction is the earliest significant biophysical effect of water stress, turgor dependent activities such as leaf expansion and root elongation are the most sensitive to water deficits. Because leaf expansion depends mostly on cell expansion, the principles that underlie the two processes are similar. Inhibition of leaf expansion results in a slowing of leaf expansion early in the development of water deficits. The smaller leaf area transpires less water, effectively conserving a limited water supply in the soil over a longer period. Reduction in leaf area can thus be considered as the first line of defense against drought (Taiz and Zeiger, 2002).

1.5.2.1.3 Osmoprotectant biosynthesis

Organic compatable solutes/osmoprotectants are low molecular weight organic compounds primarly accumulated in response to osmotic stress in diverse taxa including plants (Yancey *et al*, 1982). They are highly soluble compounds carry no net charge at physiological PH and non toxic even at higher concentrations. These molecules increase the osmotic pressure in the cytoplasm, thereby maintaining driving gradient for both water uptake and turgor pressure. Apart from osmotic adjustment,

these compounds are reported to function as scavengers for ROS, having chaperon-like activity and help in metabolic detoxification (Serraj and Sinclair, 2002). The compounds fall into several groups - amino acids (e.g. proline), quaternary ammonium compounds (glycine betaine), polyols and sugars (mannitol, Dononitil, trehalose, sucrose, fructan) (Nuccio *et al*, 1999).

Rice has two genes encoding the betaine aldehyde dehydrogenase, which catalyzes betaine aldehyde to glycinebetaine (GB), a compatible solute. However, rice cannot synthesize GB because of the lack of an upstream enzyme, the choline monooxidase (CMO), which convert a choline to a betaine aldehyde. Introductions of spinach CMO genes or the Arthrobacter pascens choline oxidase into rice plants promoted the synthesis of GB in the transgenic rice plants (Sakamoto *et al*. 1998; Shirasawa *et al*. 2006). However, only relatively small amount of GB accumulation and slight enhancement of salt tolerance of transgenic rice plants were observed in some conditions tested, probably because of low activities and/or miss-localization of the introduced enzymes (Shirasawa *et al*. 2006).

1.5.2.2 Adaptive responses to the specific ion toxicity

Plants have evolved two very different strategies in adapting to high level of sodium salts in their environments. One strategy is to exclude the salts from the leaf cells, and the other includes the salt within the leaf cells but sequestrates most of them in the vacuoles of those cells. In both cases, the end result is to maintain the cytoplasmic sodium concentration relatively low (Sen *et al*, 1995).

The sensitivity of the metabolic enzymes in both halophytes and glycophytes to high sodium level is comparable. Therefore, keeping the cytoslic K^+/Na^+ as high as possible is a basic requirement for all plants to cope with toxic effect of sodium ions (Gelnn *et al*, 1999).

1.5.2.2.1 Sodium efflux: SOS1 as a well characterized protein

The salt tolerance in rice correlates with ability to exclude Na+ from the roots and shoots, and to maintain a low cellular Na^+/K^+ ratio. The Na^+/K^+ antiporter Salt Overly

Sensitive (SOS1) is the only sodium efflux protein at the plasma membrane of plants characterized so far (Martı´nez-Atienza *et al*, 2007). At first sight, the efflux of Na+ out of individual cells is not logical in multicellular organisms such as plants, as the extrusion of Na+ could negatively impact the surrounding cells (Zhu 2003).

In Arabidopsis, Na^+ efflux is catalyzed by the plasma membrane Na^+/H^+ antiporter encoded by the previously mentioned SOS1 gene (Shi *et al*. 2003). Martı´nez-Atienza *et al*, 2007also proved the SOS1 system in rice on the level of molecular biology and biochemistry to be a highly conserved model.

As we mentioned before, it is not known in any organism how Na^+ is sensed and how the sensing leads to cytosolic ca^{++} transient. Nevertheless, it is hypothesized that SOS1 can sense Na^+ ions (Shi *et al*, 2003). As shown in Figure 5, after sensing Na^+ the level of calcium in the cytoplasm increased significantly (knight *et al*, 1997). calcium-binding protein SOS3 senses salt-elicited Ca^{2+} signal and translates it to downstream responses by interacting with and activating the protein kinase SOS2 (Sanchez-Barrena *et al*, 2007).Then, SOS2 and SOS3 regulate the expression and/or activities of various ion transporters including the plasma membrane Na^+/H^+ antiporter SOS1 (Shi *et al*., 2003; Qiu *et al*., 2002).

1.5.2.2.2 Na+ sequestration into the vacuole

the sequestration of Na+ in the vacuoles is a efficient mechanism to reduce the cytosolic Na+ concentrations (Yamagashi and Blumwald, 2005). Additionally, the sequestrated Na+ ions in the vacuoles acts as osmoticum, which help the plant cell to manipulated the water uptake under saline environment (Horie *et al*, 2012).

NHX transporters catalyze transport of Na+ into the vacuole in exchange for protons. The first plant Na+/H+ antiporter, AtNHX1, was molecularly characterized from Arabidopsis by functional genetic complementation of a yeast mutant devoid of endosomal Na+/H+ activity, and its overexpression suppressed its salt hypersensivity phenotype (Gaxiola *et al*, 1999).

Scattered evidences suggested that NHX protein can u into the vacuoles in order to regulate the pH value of the cytosol to help smooth cell expansion (Bassil *et al*, 2011).

Very recently, using reverse genetics, it was indicated that NHX1 and NHX2 (the major two tonoplast-localized NHX isoform) were highly expressed in guard cells and are very essential in stomatal function. They are responsible for K^+ uptake into the vacuoles in order to regulate cell turgor and stomatal function in Arabidopsis (Barragán *et al*, 2012).

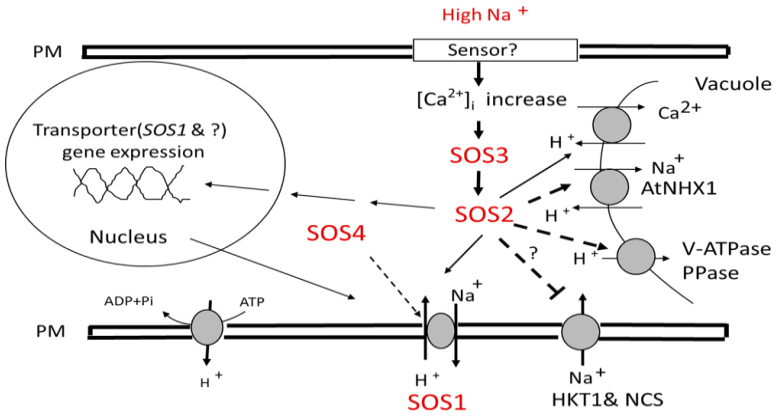

Figure 5: SOS1 system (sodium ions efflux) and NHX1 system (sodium ions sequestiration).
Source: https://ag.purdue.edu/hla/zhulab/Pages/default.aspx

1.6 Adaptive response to oxidative stress

1.6.1 What are ROS?

Reactive oxygen species (ROS), also called active oxygen species (AOS) or reactive oxygen intermediates (ROI) are the result of the partial reduction of atmospheric O2. There are basically four forms of cellular ROS, singlet oxygen ($^1O_2$*), superoxide radical ($O2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO·), each with a characteristic half-life and an oxidizing potential. ROS can be extremely reactive, especially singlet oxygen and the hydroxyl radical and, unlike atmospheric oxygen, they can oxidize multiple cellular components like proteins and lipids, DNA and RNA. Unrestricted oxidation of the cellular components will ultimately cause cell death (Mittler, 2002). Molecular oxygen is in its ground state (triplet oxygen) is essential to life on earth. It is relatively stable molecule that does not directly cause damage to living cells. However, when the triplet oxygen received extra energy or electrons, it generates a variety of ROS that will cause oxidative damage to various components of living cells (Abogadallah, 2010). Triplet oxygen has two unpaired electrons with parallel spin located in different orbitals. Upon receiving extra energy from a photosensitizer as

chlorophyll, these two electrons show anti-parallel spin, a change that substantially increase the oxidizing power of oxygen, and in that case it called singlet oxygen ($^1O_2$*) (Krieger-Liszkay, 2004). On the other hand, when the triplet ground state molecular oxygen receive an electron, it give rise to superoxide radical (O_2^-), which generates hydrogen peroxide and hydroxyl radicals through a series of chemical conversions (Apel and Hirt, 2004).

1.6.2 Production of ROS

Plants produce ROS under normal conditions essentially from photosynthesis, photorespiration and respiration. Additionally sources include NADPH oxidase, amine oxidase and cell wall bound peroxidases (Mittler *et al*, 2002). The most common ROS generated under normal conditions are superoxide and hydrogen peroxide, unlike singlet oxygen and hydroxyl radical whose production is kept at minimum levels (Jakob and Heber, 1996).

1.6.2.1 Chloroplast and ROS

During photosynthesis, energy from the sunlight is captured and transferred to two light-harvesting complexes (photosystem II and photosystem I) in the chloroplast thylakoidal membranes. A succession of redox (reduction-oxidation) reactions occurs within the electron transport chain in the light, until electrons finally reach CO_2, in the dark reactions. However, it is not uncommon that through this path other final acceptors of electrons are used, namely molecular oxygen (de Carvalho, 2008).

Singlet oxygen can be formed by energy transfer from triplet excited chlorophyll to O_2. On the other hand, the thylakoidal electron transport components on PSI side such as the Fe-S centers and the reduced thiredoxine are auto-oxydable resulting in the reduction of O_2 (Mehler reaction) thus forming superoxide anion and hydrogen peroxide. It has been estimated that 10% of the photosynthetic electron flow ‘leaked’ to Mehler reaction, and more interestingly, this ‘leakage’ is in the favor of PSI as it make it more balanced and efficient (Foyer and Noctor, 2000).

During photosynthesis there is a different pathway, called photorespiration that can also generate ROS. In fact, rubisco, the enzyme that catalyses the carboxylation of

ribulose-1, 5-bisphosphate (RuBP) during carbon assimilation, can also use O_2 to oxygenate ribulose-1, 5-bisphosphate. This reaction yields glycolate that is then transported from chloroplasts to peroxisomes where they are oxidized by glycolate oxidase and H_2O_2 is generated (Wingler *et al*, 2000).

1.6.2.2 ROS production under salinity stress

Theoretically, the plants suffer from salt stress were expected to be ROS accumulators significantly more than under unstressed conditions. According to the literatures, we tried to summaries this story in Figure 4. Mainly salt stress encourages ROS production through four main routes, 1) electron leakage to Mehler reaction, 2) photorespiration (particularly C3 plants, 3) activation of NADPH oxidase and Diamine oxidase, and 4) increasing the rate of electron leakage from respiration (Abogadallah, 2010).

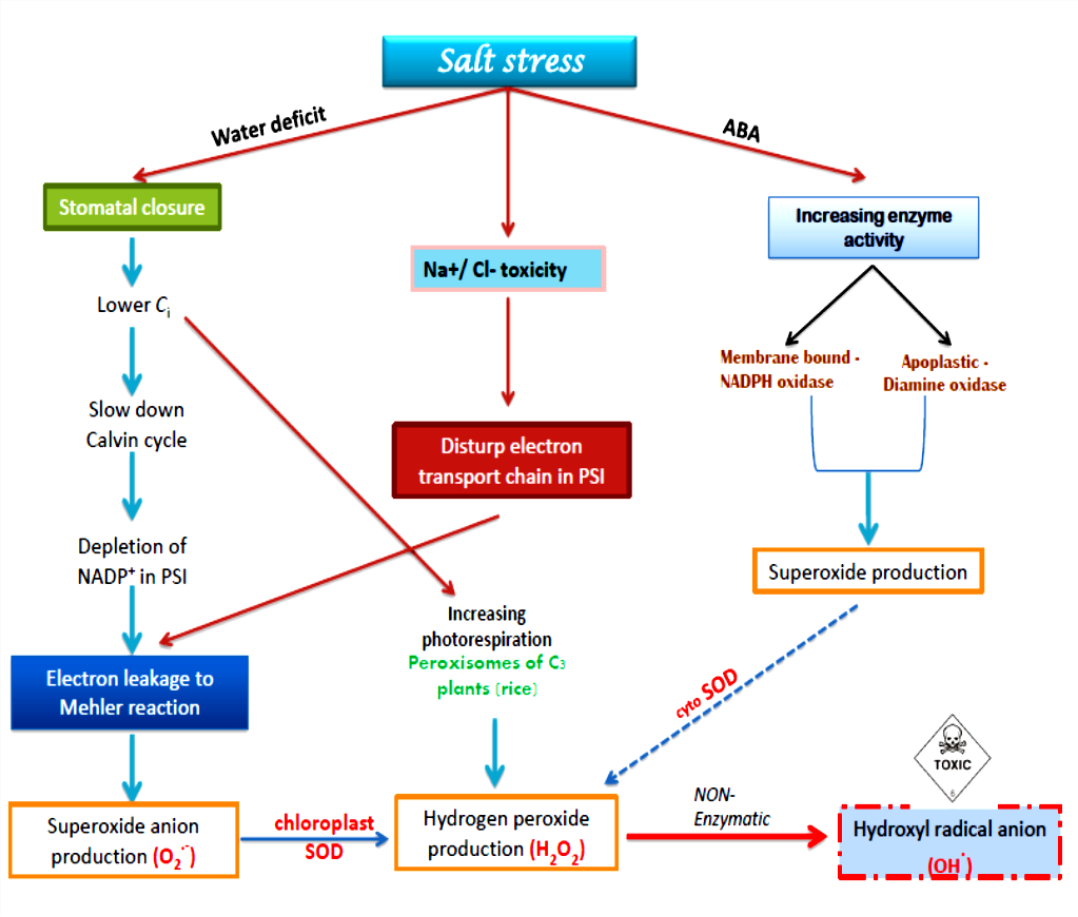

Figure 4: The routes of ROS over-production under salt stress in plants.

1.6.3 ROS scavenging

In order to cope with continuous ROS production, plants have evolved a battery of enzymatic and nonenzymatic antioxidants, which function as an extremely efficient cooperative system.

1.6.3.1 Enzymatic antioxidants

The major scavenging mechanisms include superoxide dismutase (SOD), enzymes and metabolites from the ascorbate-glutathione cycle, and catalase (CAT). They are located throughout the different compartments of the plant cell, with the exception of catalase that is exclusively located in peroxisomes. SOD is the front-line enzyme in ROS attack

Since it rapidly scavenges superoxide, one of the first ROS to be produced, dismutating it to oxygen and H_2O_2 (Bowler *et al*, 1992). However, this reaction only converts one ROS to another, and H_2O_2 also needs to be destroyed since it promptly attacks thiol proteins (Foyer and Noctor, 2000). The major enzymatic cellular scavengers of H_2O_2 are catalase and ascorbate peroxidase (APX) (Willekens *et al*, 1997). They have however different affinities for this ROS and seem to have different cellular roles in H_2O_2 scavenging. In fact CAT does not need a reductant to scavenge H_2O_2 making it reducing power-free, whereas APX needs a reductant, ascorbate (de Carvalho, 2008).

1.6.3.2 Non-enzymatic antioxidants

Plants evolved another safe line of antioxidants; it is the chemical or non-enzymatic antioxidants. Natural antioxidants occur in all parts of the plant. These antioxidants include carotenoids, vitamins, phenols, flavonoids, dietary glutathione, and endogenous metabolites. Ascorbic acid is synthesized in higher plants and is one of the key products of D-glucose metabolism. It affects plant growth and development, and plays an important role in the electron transport system (Tuna *et al*, 2010). The antioxidants prosperities of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper, and inhibition of enzymes responsible for free-radical generation (Benaventa-Garcia *et al*, 1997).

Other than as an osmolyte, now Proline is considered as a potent antioxidant and potential inhibitor of programmed cell death. Therefore, Proline can now be regarded as nonenzymatic antioxidants that microbes, animals, and plants require to mitigate the adverse effects of ROS (Gill and Tuteja, 2010). Glutathione (GSH), the tripeptidec-glutamylcysteinyl-glycine,is the major source of non-protein thiols in most plant cells. GSH plays an important role in the response of plants to environmental stresses, including oxidative stress, xenobiotics, and some heavy metals (Ruiz and Blumwald, 2002).

1.6.4 ROS as signals

Reactive oxygen species play a multitude of signaling roles in different organisms from bacteria to mammalian cells. They were initially thought to be toxic byproducts of aerobic metabolism, but have now been acknowledged as central players in the complex signaling network of cells (Mittler *et al*, 2011).

Several possible advantages come to mind when considering the use of ROS as signaling molecules. These include the capacity of the cell to rapidly produce and scavenge different forms of ROS in a simultaneous manner, enabling rapid and dynamic changes of ROS levels (caused by simply tiling the balance between cellular and scavenging rate). Another advantage could be a tight control over the subcellular localization of ROS signals in the cells, such as certain organelles or membranes (Monhausen *et al*, 2009 and Takeda *et al*, 2008).

An additional signaling advantage of ROS is that different forms of ROS exist, with significantly different molecular prosperities. For example, superoxide anion is a charged molecule under most physiological conditions and could not passively transfer across a membrane. By contrast, superoxide could be transformed to hydrogen peroxide which is water soluble and can travel long distance in a short time through water channel or through passive transport (Miller *et al*, 2010).

1.6.4.1 ROS relation to the other signaling network (H_2O_2 as an example)

The known downstream events modulated by H_2O_2 are calcium mobilization, protein phosphorylation and gene expression. Changes in $[Ca^{++}]_{cyt}$ have been reported in many abiotic and biotic signal transduction pathways. It has been shown that ROS

induces an increase in $[Ca^{2+}]_{cyt}$ by the activation of hyperpolarization-dependent Ca^{2+}-permeable channels in the plasma membrane of Arabidopsis guard cells (Pei *et al*, 2000).A good example for ROS-activated signaling network is the mitogen-activated protein kinases (MAPK) cascades. Many different MAPKs cascades can be activated following ROS accumulation. Recently, a unique short cut in MAPK pathways has been found for MEKK1- it can interact directly with the transcription factor WRKY53 and phosphorylates it, therefore the increasing of WRKY53 DNA-binding activity and allows the immediate induction of stress and defense- related target genes by bypassing of downstream kinases (Miao *et al*, 2007 and Petrov and Breusegem, 2012).

1.7 Jasmonic acid (JA): it is not just a scent!

Jasmonic acid and its fragment methyl ester, methyl jasmonate (MeJA), a volatile constituent of essential oil of Jasmine, rosemary and many other flowers. Those biologically active compounds and their derivatives collectively called as jasmonates, became of interest of plant physiologists in the early 1980s when they were shown to retard the growth of roots and coleoptiles and to promote leaf senescence (Srivastave, 2001).JA is an important developmental regulator involved in seed germination, primary root growth, flowering, fertilization and senescence (Feussner and wasternack, 2002). The ability of plants to synthesis and perceive JA is absolutely essential for the convert development and release of pollen in Arabidopsis (Feys *et al*, 1994).

1.7.1 Activation of jasmonic acid signaling by repressor removal

Many plant processes are controlled by repressors of downstream transcriptional networks, and the degradation of these repressors under external stimuli and by plant hormones provides a rapid regulatory trigger system. The involvement of protein degradation pathways in JA signaling became apparent after the identification of the COI1 gene encoding an F-box protein with Leu repeats (Xie *et al*., 1998). The core event of JA perception is illustrated in figure 6 as reported by Ballaré, 2011. The perception of jasmonosyle-iso-leucine (JA Ile), the bioactive amino acid conjugate of jasmonic acid, is achieved by the ubiquitin ligase SCF^{COI1} complex. When the Fbox protein CORONATINE-INSENSITIVE 1(COI1) recognizes JA-Ile, it triggers the ubiquitination and subsequent proteosomal degradation of JASMONATE ZIM DOMAIN

(JAZ) proteins. The degradation of JAZ proteins relieves JAZ-mediated repression of gene expression, leading to the activation of JA responses (Chini *et al*, 2007 and Yan *et al*, 2007).

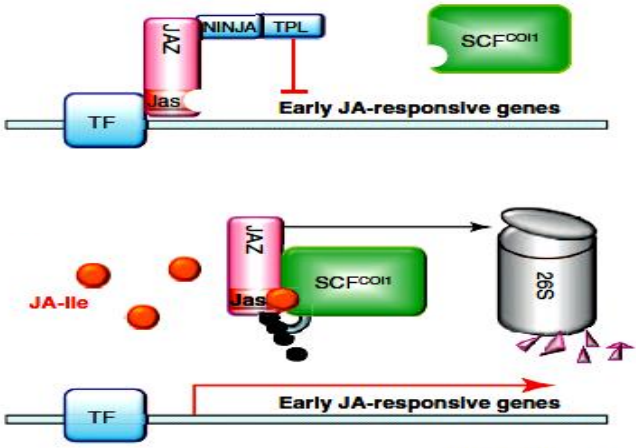

Fig 6: Activation of JA pathway through repressor removal. Source: Ballaré, 2011(part of it).

1.7.2 Integration of JA with plant stress

1.7.2.1 Jasmonates induce defense responses

JAs were first connected with defense responses through the regulation of the expression of digestive proteinase inhibitors in the tomatos (Farmer and Rayan, 1990). Two decades after that exciting discovery, it is now firmly established that JA is a key cellular signal involved in the activation of immune responses to most insect herbivores and necrotrophic microorganisms (Ballaré, 2011).

In response to wounding stress, the plants trigger higher rate JA-biosynthesis due to the activation of JA-biosynthsis enzymes such as AOC (Allien oxide cyclase) as a results of sequential action of the 18-aa peptide systemine (Wasternack, 2007). The accumulation of JA leads to enhancing the immunity system of injured plants against the attacking herbivores through possible strategies, as a systemic signals that result in a systemic expression of genes encoding proteinase inhibitor (PINs) and other folair compounds with negative effect on the herbivore performance and growth. Additionally some compounds as hydrogen peroxide which could cause toxic acceft on the attacking herbivore (Narváez-Vásquez and Ryan, 2004).

1.7.2.2 JA and Salinity stress

It has been demonstrated that exogenous JA may be involved in the defense not only during wounding and pathogen stress, but also during salt and water stress (Kang *et al*, 2005). Nevertheless, little is known about the involvement of JA in salt stress-signaling (Shahzad, 2011).Several studies have investigated biological relevancies of JA signalling in salt stress in rice. Interestingly, higher endogenous JA contents were observed in salt-tolerant cultivar rice than in salt-sensitive cultivar (Kang *et al*. 2005). Similar results were observed in tomato plants by Pedranzani *et al*, 2003. In addition, MeJA level was increased by high salt stress in rice (Moons *et al*. 1997), supposing that high accumulation of JA in rice could be an effective protection against salt stress. Consistently, exogenous JA treatment dramatically reduced the Na^+ ions in salt-tolerant cultivar rice (Kang *et al*. 2005).The cross talk between JAs and other hormones may increase the mystery of JA role within adaptation mechanisms under salinity stress. MYC2 is a well known transcription factor for JA and ABA as well (Kazan and Manners 2012). Some reports showed that the exogenous addition of JA caused an elevation in the endogenous level of ABA in rice (Seo *et al*, 2001). However, the antagonistic roles of JA in ABA-mediated regulations of salt-stress related gene expressions have been also reported in rice root. JA treatment effectively reduced the ABA-mediated up-regulation of OsLEAs in rice root. Furthermore, JA-inducible genes were not stimulated in the presence of JA and ABA (Moons *et al*. 1997). Taken together, this implies the involvement of different regulation mechanisms in JA and ABA-mediated responses to salt stress (Kumar *et al*, 2013).

1.8 Scope of this study

Soil Stalinization is a worldwide threatening problem that causes a huge reduction in the universal agricultural crops yield, more than 20% of all irrigated land on earth affected by Stalinization. Rice (Oryza sativa) the world's most important cereal crop, as it is the primary source of food and calories for about half of mankind (Khush, 2005), but unfortunately it is very sensitive to salinity stress specially at seedling stage, its height, root length, emergence of new roots, and dry matter decrease significantly under salinity (Pearson *et al* 1966, Akbar and Yabuno 1974). In addition, it has developed

into the model species for the Graminea (such as wheat, maize, and barley) because it is diploid and harbours the smallest genome of all Graminea. Due to the conserved genomic organization across the Graminea, data from rice can be directly transferred to other Graminean species.The adaptive response of salt stressed plants is strongly depending on chemical signals in order to regulate growth and development in such unfavorable conditions. Jasmonic acid (JA) is among these signals, however, little is known about its function and its cross talk to other plant hormones which are known to be involved in salt stress adaptation as Abscisic acid (ABA). The described study in this thesis aims to investigate more about the role of JA in response to salinity stress in rice as a model plant for monocots and as a very important universal stable food crop. The output of this study might help in Identification of abiotic stress-inducible key genes from rice that could be used as marker genes for future breeding programs for drought and /or salt stress tolerance (marker-assisted breeding) with respect to the role of JA as a crucial plant hormone.

One of the challenges that facing investigations of jasmoantes role in salinity stress physiology and biochemistry in rice is the unavailability of well-characterized biosynthetic rice mutants. Riemann *et al*., 2013 successfully isolated and characterized two Jasmonate-deficient mutants (cpm2 and hebiba) which were used in this study. Homozygous seedlings plants of the two JA-biosynthesis mutants (cpm2 and hebiba) were compared to their wild type background rice cultivar-Nihonmassari on the level of morphology, biochemistry and molecular biology in order to find out more knowledge about the involvement of jasmonates in salt stress response in rice. cpm2 or coleoptile photomorphogenesis 2 is a specific mutant of ALLENE OXIDE CYCLASE (AOC), carrying an 11 bp deletion within the first exon. The mutant was rescued by complementation with the OsAOC wild type allele. While hebiba was isolated from a screen of plants defective in photomorphogenesis as a putative red blind candidate, the AOC and STZ1 (Salt Tolerance Zinc-finger transcription 1) genes with others are deleted in hebiba plantlets, both of the mutants exhibited a distinguishable phenotype in light or dark conditions.

The outline of this work is started by the phenotypic examination of JA-mutants and WT when the salinity stress was triggered. The phenotyping approach was done on the level of root length and salinity stress appeared symptoms on second and third leaves. Furthermore, the uptaken sodium ions in roots and shoots tissues of the plants was also quantified in order to give a possible connection to the observed phenotype. To better understand how both of cpm2 and hebiba, as JA-biosynthesis mutant lines, will handle the adaptive response to salt stress comparing to nihonmassari (WT), their responses to the oxidative stress which is born after high salt stress (100 mM) were selected as a comparing scenario. The damage of oxidative stress on the plasma membrane was evaluated through MDA (malodialdehyde) levels; also the level of H_2O_2 was measured. On the other hand, the quantities of some antioxidants compounds as soluble proline, total phenolics and total flavonoids were determined. The level of antioxidative power was estimated through the scavenging activity of both of non-enzymatic and enzymatic antioxidant machinery. For the non-enzymatic antioxidants, the following activities were measured: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, superoxide anion scavenging assay (SOSA), hydroxyl radical scavenging assay (HRSA) and hydrogenperoxide scavenging assay (HPSA). For the enzymatic response to oxidative stress, the activities of the following antioxidants enzymes were estimated: superoxide dismutase (SOD), catalse (CAT), ascorpate peroxidase (APX), peroxidases (POD), glutathion reductase (GR) and glutathion-S-transferase (GST). For an explanation to the described phenotype, the expression profile of some genes candidate as OsNHX1, OsSAT, OsOXO4, OsJAZ13, OsNCED5 and OsNR were measured through real time PCR. Finally, the endogenous level of some plant hormones was measured as JA, JAIle, OPDA, ABA and NO; the later one was estimated histochemically in the guard cells of stomata of rice leaves.

2- Materials and Methods

2.1 Plant materials, growth and stress conditions

In this study Oryza sativa L. ssp. japonica cv. Nihonmasari was used as the wild type. The two mutant lines cpm2 and hebiba were generated in the same cultivar (Riemann *et al*. 2013). The caryopsis were dehusked and surface sterilized by incubating the seeds in 70% ethanol for 1 min then washed briefly 2 times with ultrapure water. Subsequently, the seeds were incubated in a sodium hypochlorite solution containing approximately 5% of active chlorine for 30 min followed by 5 washing steps in ultrapure water. The surface sterilized seeds were kept in ultrapure water overnight in the refrigerator before sowing.

The seeds were sown on 0.5% phytoagar medium (Duchefa, Netherlands) and incubated for 10 -12 days in a culture room at 25°C under continuous light with an intensity of 120 µmol photon $m^{-2}s^{-1}$. After 10-12 days, the well grown seedlings were installed in sterilized floating racks and moved to a glass container containing ultrapure water as control or a solution containing NaCl to cause salt stress, respectively. The treatments lasted three days. After three days, the shoots of the control and the stressed plants were harvested and kept in liquid nitrogen, then stored in -80C° to be used in the anti-oxidants non-enzymatic, enzymatic measurements and for gene expression analysis.

2.2 Analysis of root elongation

Root elongation was evaluated as the mean of the seminal root length of seedlings raised in darkness at 25C° for 7 days. The seeds were surface sterilized as described above, and sown on 0.5% phytoagar medium with different concentrations of NaCl (0, 7.8, 15.6, 31.3, 62.3, 125 and 250 mM). The seedlings were scanned and the root length was measured using Image J.

2.3 Sodium ion content

After salinity stress, the seedling were washed with deionized water perfectly and allowed to be dried in 80C° oven for three days, then the dried shoots and roots separated, crushed to fine pieces and their weight was recorded. Each sample was digested in concentrated Nitric acid and boiled for three hours in a boiling water bath using a disposable digestion tube. Na^+ contents in both roots and shoots were measured using atomic absorption spectroscopy (AAS).

2.4 Estimation of chlorophyll content

Total chlorophyll, chlorophyll a and chlorophyll b contents were determined following the method of Arnon (1949). 100 mg of leaves was weighed from each sample and homogenized with acetone. The extract was filtered through whatman No.1. filter paper and washed 2-3 times with 80 per cent acetone. The final volume of the extract was made up to

25 ml. The absorbance of the extract was read at 645, 652 and 663 nm in spectrophotometer and for blank 80 per cent acetone was used.
The chlorophyll content was then estimated using the following formula.

$$\text{Chlorophyll a (mg/g r. wt.)} = 12.7\,(A663) - 2.69\,(A645) \times \frac{V}{100 \times w \times a}$$

$$\text{Chlorophyll b (mg/g r. wt.)} = 22.9\,(A645) - 4.68\,(A663) \times \frac{V}{100 \times w \times a}$$

Total chlorophyll was rechecked using the formula.

$$\text{Total Chlorophyll (mg/g r. wt.)} = 27.8\,(A652) \times \frac{V}{100 \times w \times a}$$

Where,A = Absorbance at different wavelength, V = Final volume (ml), w = Fresh weight of the sample (g), a = path length (1 cm)

2.5 Determination of lipid peroxidation level

Lipid peroxidation of shoots was estimated by the level of MDA (Malondialdehyde) using the thiobarbituric acid (TBA) method as described by Heath and Packer (1968). Briefly, 500mg of rice shoots were homogenized using mortar and pestle in 0.1% TCA (Trichloroacetic acid, w/v), 1 ml. The homogenate was then centrifuged at 10,000xg for 20 min, and 0.5 ml of the supernatant was added to 1 ml of 0.5% TBA in 20% TCA. This mixture was allowed to be heated in a boiling water bath for 1 hour. The reaction was stopped by transferring the tubes to an ice bath for 10 min, and then the tubes were centrifuged for 10 min at 10,000xg. The absorbance of the supernatant was recorded at 532 nm and 600 nm. The value of the non-specific absorption at 600 nm was subtracted. The amount of MDA-TBA complex (red pigment) was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

2.6 Estimation of aqueous peroxide level

Steady state level of H_2O_2 in the shoots of control and salt-stressed rice seedling were measured using the FOX1 method (Ferrous Oxidation with Xylenol orange) (Wolf, 1994) with some modifications. Leaves (70 mg fresh weight) were perfectly ground in 5 ml of 5% TCA containing 100 µg of active charcoal. The mixture was filtered using No.1 filter paper (whatman), and then a measured volume of the filtrate was incubated with FOX-1 reagent (100 µM xylenol orange, 250 µM ammonium sulphate, 100 mM sorbitol and 25 mM H_2SO_4) for 30 min. the absorbance was recorded at 560 nm. The values of aqueous peroxide were referred to as micromoles H_2O_2 using a standard curve.

2.7 Evaluation of antioxidants amounts

2.7.1 Soluble proline estimation

Free proline accumulation was monitored according to Bates *et al*. (1971). Briefly, 200mg of leaves tissues were homogenized by mortar and pestle containing small amount of quartz sand. The homogenate was filtered through filter paper No.1 (Whatman). The filtrate was centrifuged for 10 min at 10,000xg at room temperature. 1 ml of the supernatant was treated with 2 ml of reaction buffer (1ml glacial acetic acid and 1 ml of ninhydrine reagent), and mixed well. The reaction was heated in a boiling

water bath for 1 hour, and then cooled to room temperature gradually. The absorbance was recorded at 520 nm. Soluble proline content was expressed as micromoles proline per gram fresh weight according to a standard curve.

2.7.2 Amount of total polyphenols

The total phenolic content was determined according to the Folin-Ciocalteu method (Singleton and Rossi, 1965). The reaction mixture was composed of 0.1 mL of methanolic extract, 7.9 mL of distilled water, 0.2 mL of the Folin-Ciocalteu's reagent (Sigma, Germany), and 1.5 mL of 20% sodium carbonate. The resultant solution was mixed and allowed to stand for 2 hours. The absorbance was measured at 765 nm in a Shimadzu UV- Spectrophotometer. The total phenolic content was determined as gallic acid equivalents (GAE)/gram dry weight.

2.7.3 Amount of total Flavonoids

Total flavonoids content was measured by the aluminum chloride colorimetric assay (Zhishen *et al*, 1999). An aliquot of methanolic extract of the samples (250ul) or the standard solution (different concentration of quercetine in ug/ml) was added to 10 ml of volumetric flask containing 4 ml ddH2O. to the flask was added 300 ul of 5% NaNO2. After 5 min, 300ul of 10% AlCl3 was added. At 6^{th} min, 2 ml of NaOH was added and total volume was made up to 10 ml with dd H2O. the solution was mixed well and the absorbance was measured against prepared reagent blank at 510nm. Total flavonoids content of rice leaves methanolic extract was expressed at mg quercetien per gram dry weight.

2.8 Non-enzymatic antioxidants scavenging activity

2.8.1 Free radical scavenging activity (DPPH scavenging activity)

Methanolic extract of control and salt-stressed rice seedling shoots were prepared. The plants were harvested and freeze dried. Subsequently 300 mg of dried plant soot powder from each sample was homogenized in 20 ml of HPLC grade methanol, and the mixture was shaken (150rpm) in darkness for 2 hours, and stored overnight at -20°C. The homogenate was filtered using filter paper No.2 (Whatman). Rotating evaporator

was used to evaporate the methanol (250 bars at 45°C). The extract was dissolved in HPLC grade methanol again to give concentration of 10 mg/ml extract.

In order to measure the antioxidant ability of the rice shoot extract, DPPH (2, 2-diphenyl-1-picrylhydrazyl), a stable free radical, was used according to Goffman and Bergman (2004). Rice leaves extract (100 µl) with different concentrations (37-370 µg extract /ml reaction) were added to 900 µl of freshly prepared DPPH methanolic solution (80 ppm). The reaction mixture was mixed well and incubated in darkness at room temperature for 30 min. The antiradical efficiency of the rice leaf extract was determined by monitoring the reduction in the absorbance (515 nm) of the methanolic solution of DPPH. DPPH inhibition percentage (I %) was calculated using the following formula:

$$\frac{Ab_{cont} - Ab_{sample}}{Ab_{cont}} \times 100$$

Where $Ab_{cont\ is}$ the absorbance value at 515 for the DPPH mix containing 100 µl methanol instead of 100 µl extract sample. The IC_{50}% value for each sample was calculated to determine the amount in micrograms of extract sufficient to scavenge 50% or half of the DPPH radical substance. Hence a lower IC_{50}% corresponds to a higher antioxidant activity. Butylated hydroxyanisole (BHA), as a very efficient antioxidant, was used as an internal experimental control.

2.8.2 Specific ROS scavenging activity estimation

2.8.2.1 Plant crude aqueous extracts preparation

Rice leaves were collected from both control and salt stressed plants and washed gently several times with Millipore water. The washed leaves were grounded using mortar and pestle in 5ml warm Millipore water (80Cº), then the homogenate was mixed with 50 ml of Millipore water and allowed to be stirred for 3 hours. After that, the aqueous mix was filtered through filter paper No2 (Whatman). This process repeated one more time and the combined filtrate was freeze dried. The produced yellowish solid

crude extract were kept in -20Cº to be used for testing its scavenging activity of superoxide anion, hydrogen peroxide and hydroxyl radical.

2.8.2.2 Superoxide anion scavenging activity

Measurement of superoxide scavenging activity was done based on the method described by Zhishen *et al*, 1999 with slight modifications. Superoxide radicals are generated in riboflavin, methionine, illuminate and assayed by the reduction of nitroblue tetrazolium (NBT) to form blue formazan ($NBT2^{+}$). All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using fluorescent lamps (20 W). The total volume of the reactant mixture was 3 ml and the concentrations of the extract in the reaction mixture were 25, 50, 100, 150 and 250 µg/reaction mixture. The reactant was illuminated at 25°C for 40 min. The photochemically reduced riboflavin generated O2 this reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank. The absorbance was measured at 560 nm. The extract was added to the reaction mixture, in which O2 was scavenged, thereby inhibiting the NBT reduction. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The inhibition percentage of superoxide anion generation was calculated by using the following formula:

$O2^{\cdot -}$ scavenging effect (%) = $[(A_0 - A_1)/A_0)] \times 100$

Where A_0 is the absorbance of the control, while A_1 is the absorbance of sample and standard. The reaction mixture with water instead of sample is referred as the control. IC_{50} value is concentration of sample in ug/reaction mixture required for the scavenging of 50% of introduced superoxide anion in the reaction mixture and it is calculated from the inhibition % curve.

2.8.2.3 Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging activity the H_2O_2 scavenging activity of the extract was determined by the method of Ruch *et al*. (1989). The extract at different concentration (60, 120, 240, 300 and 420 µg/reaction mixture) was dissolved in 3.4 ml of 0.1 M phosphate buffer pH 7.4) and mixed with 600µl of 43mM solution of H_2O_2. The

absorbance value of the reaction mixture was recorded at 230 nm and BHT was considered as the standard. The percentage scavenging of H_2O_2 was calculated as follows: Inhibition of H_2O_2 (%) = 1-Sample 230nm/Control 230nm x 100, where, Sample 230nm was absorbance of the sample and Control 230nm was the absorbance of control.

2.8.2.4 Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was measured by the studying the competition between deoxyribose and test compound for hydroxyl radical generated by Fe+3-Ascorbate-EDTA-H_2O_2 system (Fenton reaction) according to the method of Kunchandy and Rao (1990). The hydroxyl radicals attacks deoxribose and eventually results in TBARS formation.

The reaction mixture contained 0.8 mL of phosphate buffer solution (50 mM, pH 7.4), 0.2 mL of a sample of different concentrations (25, 50, 100, 150 and 250 µg/reaction mixture) 0.2 mL of EDTA (1.04 mM), 0.2 mL of FeCl3 (1 mM), and 0.2 mL of 2-deoxyribose (60 mM). The mixtures were kept in a water bath at 37 °C and the reaction was started by adding 0.2 mL of ascorbic acid (2 mM) and 0.2 mL of H_2O_2 (10 mM). After incubation at 37 °C for 1 h, 2 mL of cold thiobarbituric acid (10 mg /ml) was added to the reaction mixture followed by 2 mL of HCl (25%). The mixture was heated at 100 °C for 15 min and then cooled down with water.

The absorbance of solution was measured at 532 nm with a spectrophotometer. The hydroxyl radical scavenging capacity was evaluated with the inhibition percentage of 2-deoxyribose oxidation on hydroxyl radicals. The scavenging percentage was calculated according to the following formula:

Scavenging percentage %= $[A_0 – (A_1 – A_2)] \times 100/A_0$ where A_0 is the absorbance of the control without a sample. A1 is the absorbance after adding the sample and deoxyribose. A_2 is the absorbance of the sample without deoxyribose.

2.9 Protein extraction and antioxidant enzyme activity measurement

For estimating the activity of catalase (CAT), Peroxidase (POD), Glutathion reductase (GR), glutathione-S-transferase (GST) and superoxide dismutase (SOD), leaves of 10

days old seedlings were homogenized in 1 ml of ice cold extraction buffer according to Venisse *et al*. (2001). In brief, the shoots of 4 seedlings were homogenized in cold extraction buffer (50mM sodium phosphate buffer (pH 7.5), 1 mM polyethyleneglycol, 1 mM phenylethylemethylesulfonylfluoride, 8% (w/v) polyvinylpyrolydone and 0.01% (v/v) triton X-100). The mixture was centrifuged at 14,000 rpm for 30 min at 0 °C; the filtrate was used in total protein estimation according to Bradford (1976). For ascorbate peroxidase (APX), the same procedure was followed but using the extraction buffer of Nakaro and Asada (1981).

For CAT (EC1.11.1.6), the activity was estimated spectrophotometrically by following the disappearance of hydrogen peroxide at 240nm (extinction coefficient 39.4 mM^{-1} cm^{-1}). Calculations of CAT activity were made according to Abei (1980). One unit of CAT activity was defined as the amount of enzyme required to oxidize 1 µmol of H_2O_2 per minute (Wegdert and Cullen, 2009). APX (EC 1.11.1.11) activity was measured according to Nakano and Asada (1981) by monitoring the rate of H_2O_2-dependent oxidation of ascorbate at 290 nm (E=2.8 mM^{-1} cm^{-1}). The reaction mixture included 50mM potassium phosphate buffer (pH7), 0.5 mM ascorbic acid, 0.1 mM H_2O_2, 1 mM EDTA and enzyme extract.

GR (EC 1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient of 6.2 mM^{-1} cm^{-1}) according to Halliwell and Foyer (1978). Fifty microliters of leaf extract was added to 1 mL of the reaction mixture. The mixture consisted of a solution of 0.2 mM Tris/HCl buffer (pH 7.8) containing 3 mMEDTA, 0.2 mM NADPH, and 0.5 mM oxidized glutathione.

GST (EC 2.5.1.18) activity was determined by measuring the formation of the conjugate reaction product (extinction coefficient of 9.6 mM^{-1} cm^{-1}) at 340 nm using 1-chloro-2, 4-dinitrobenzene and glutathione as substrates (Mauch and Dudler, 1993). Fifty microliters of leaf extract was added to 1 mL of the reaction mixture. The mixture consisted of a solution of 0.1 M potassium phosphate (pH 6.5), 3.6 mM reduced glutathione, and 1 mM1-chloro-2, 4-dinitrobenzene.

POX (EC 1.11.1.7) activity was measured by following the increasingA_{470} due to the formation of tetraguaiacol (extinction coefficient of 26.6 mM $^{-1}cm^{-1}$) as described by Chance and Maehly (1955). Fifty microliters of enzyme source (leaf extract diluted in 50 mMsodium phosphate 1:5 for pear extract or 1:10 for tobacco extract) was added to 2 mL of the reaction mixture. The mixture consisted of a solution of 50 mM sodium acetate buffer (pH 7), 25 mM guaiacol, and 25 mMH_2O_2.

For SOD (EC 1.5.1.1) activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium at 560nm (Beauchamp and Fridovich, 1971). The reaction mixture (3 ml) consisted of 50 mM Na-phosphate buffer (PH 7.8), 13 mM L-methionine, 75 µM NBT, 10 µM EDTA-Na2, 2.0 µM riboflavin and 0.3 ml enzyme extract. The test tubes containing reaction mixture were weighed for 10 min under 4,000 lx at 35°C. One unit SOD activity was defined as the amount of enzyme required to cause a 50% inhibition of the rate of NBT reduction measured at 560 nm.

2.10 Gene expression profiling

2.10.1 Total RNA extraction

Total RNA isolation, reverse transcription and quantitative real-time PCR (qPCR) were conducted according to Svytyna *et al*. (2013). In brief, total RNA was isolated from the shoots of control and salinity stressed plants (100 mM NaCl, 24 and 72 hrs) using the InnuPrep plant RNA kit (Analytika Jena RNA kit) according to the manufacturer's instructions.

2.10.2 cDNA synthesis

The cDNA synthesis was performed with Dynamo cDNA synthesis kit (Finnzymes, Finland) using total RNA as a template. In this work, 1000 ng of high quality extracted total RNA was used for quantitative (real time) polymerase chain reaction (RT-PCR). The cDNA synthsis procedure consists of adding 1ug of RNA to 1ul of oligo dT (100 ug/ml) primers, 2ul of RT buffer containing dNTPs of 10mM, 2ul of M-MuLV RNaseH reverse transcriptase (2000U/ul), and the mixture filled with nuclease free water to be

20ul. After a brief mixing and centrifugation (10s), the mixture incubated at 25Cº for 10 min, 37Cº for 30 min, 85Cº for 5 min, and finally cooling at 4Cº.

2.10.3 Real-Time PCR

Real time (qPCR) was performed on the Opticon 2 system (Biorad, USA) as follow: 95Cº for 3 min, and 40 cycles (95Cº for 15 s and 66Cº for 30s). Data were exported from the Opticon cycler and imported as Opticon Monitor (Biorad, USA). The primer sequences for the genes of interest are listed in Table 1. To compare the transcript levels between different samples the 2 -ΔΔCt method was used (Livak and Schmittgen, 2001). First, the difference in the cycle threshold (Ct) values between the geometric means of two endogenous control genes, β-actin and EF(TU) a target gene was calculated with or without treatment, $(\Delta Ct_{gene\text{-}endoCont})_{treated}$, $(\Delta Ct_{gene\text{-}endoCont})_{non\text{-}treated}$. Then, the difference between these values was calculated as follows: $\Delta Ct_{treated\ -\ non\text{-}treated} = (\Delta Ct_{gene\text{-}endoCont})_{treated} - (\Delta Ct_{gene\text{-}endoCont})_{non\text{-}treated}$. Finally, to determine the ratio of expression levels in treated sample versus non-treated sample, we used Qr formula as follows: $Qr = 2^{-\ Ct\ treated\ -\ non\text{-}treated.}$

2.11 Hormones level estimation

2.11.1 Endogenous level of ABA, OPDA, JA and JA-Ile

OPDA, JA, JA-Ile and ABA were quantified simultaneously using a standardised UPLC-MS/MS based method according to Balcke *et al*. (2012) using $^2H_5$-OPDA, $^2H_6$-JA, $^2H_2$-JA-ile, and $^2H_6$-ABA as internal standards.

2.11.2 In situ NO production estimation

For determination of NO production in leaves segments, 10-days-old rice seedlings leaves were immersed in 10uM of the cell permeable probe DAF-FMDA (Sigma D2321-1MG) in Tris-HCl buffer 100mM, PH 7.5 for 30 min in the dark. Then leaf segments were made using sharp clean scalpel then the segments were washed three times with fresh loading buffer. After that the leaf segments were examined by confocal fluorescent microscope (DAF-FM DA excitation 490mm, emission 525 nm) in LSM 710 NLO (Carl Zeiss, Germany).

Gene name	Accession No.	Forward(5'-3' prime)	Reverse(5'-3' prime)
OsNHX1	Os07t0666900	TGTGCTCCGACAACCTGTAA	TACATGCAGGGTGGCAACTA
OsSAT	Os05t0533500-01	TTGATTGGCAGGAAGAACG	TGGTGTAGTCCGACCACTGT
OsOXO.4	LOC_Os03g48750	AATAAACTTGTCGTCGTCGCCATC	GGCGCACTTACAAAATACC
OsTIFY11e	Os10g25230	CGTGAGGATGCTTATTATGCTTG	CCAATGAAATTATATGATCCCTAGC
OsNCED5	Ay838901	ACATCCGAGCTCCTCGTCGTGAA	TTGGAAGGTGTTTGGAATGAACCA
OsNR1	Os02g0770800	TCAAAGTTTGCAGCTCTCTCG	AAATGAACCATGTGCACAACC
β-actin	AK101613	ATGCCATTCTTCTCCGTCTT	GCTCCTGCTCGTAGTC
EF(TU)	Os02t0595700	CTTGATGCCACATGGAATTG	TTGTCAAGCAAGCAAACCAC

Table 1. The sequences of forward and reverse primers for the genes of interest and the two genes used in the normalization.

3- Results

The performed experiments in this study described the comparison of rice JA-biosynthesis mutants (cpm2 and hebiba) to their wild type background rice cultivar (Nihonmassari) on the level of morphology, physiology and molecular biology. Surprisingly, the phenotype of JA-mutants appeared less stress damage symptoms in second and third leaves, and its roots were longer under salt stress. Interestingly, JA-mutants plants accumulated less Na^+ ions in its leaves than WT. On the level of oxidative damage parameters (MDA and H_2O_2), their levels found to be higher in WT leaves. Nevertheless, the soluble proline level –an antioxidant and osmoprotectant- was lower in the mutants. Furthermore, it was observed that the crude extract of the mutants efficiently detoxified the in vitro produced reactive oxygen species (ROS) more than WT extract. The antioxidant enzymes activities profile showed that Superoxide dismutase (SOD) and Peroxidases (POD), Glutathione Reductase (GR) and Glutathion-s-transferase (GST) were enhanced in mutants' shoots than WT. Gene expression profile of some genes candidates using quantitative PCR was done. The mutants showed significantly lower level of OsNHX1 and OsOXO4 transcripts in relation to WT, while the relative folds of expression of OsNCED5 and OsNR genes were higher in the mutants. The endogenous levels of JA, JA-Ile and OPDA were enhanced in response to salt stress in case of WT only but not changed in the mutants comparing to control conditions. JA-mutants produced comparable ABA in response to salt stress comparing to WT. Furthermore, it is found that the stomatal guard cells of JA-mutants leaves produced more NO than WT.

3.1 JA–mutants showed salt less-sensitive phenotype under salinity stress

The phenotyping approaches in plant stress physiology is a fundamental practical step as it should has a basic link to others experimental and practical investigations. Therefore, in order to check the effect of salinity stress on the morphological characters of both of JA-mutants and WT plants we have applied salt stress to rice plants at a very high concentration for a short-term (285mN NaCl for 24hrs, referred to as short-term) and at a high concentration for an extended period (100mM for 72 hrs, referred to as

mid-term) to 10 days old seedlings. For both stress treatments, we could observe a less salt-sensitive phenotype in both, hebiba and cpm2 leaves. Similar to the wild type mutant leaves wilted and the tissue became necrotic (Figure 7 A, B and C). However, while in the short-term exposure at high concentrations the wild type leaves were entirely rolled as a response to salt stress, the mutant leaves remained unfolded (Figure 7 A). The second leaf of the wild type was necrotic almost entirely after mid-term salt exposure the second mutant leave showed this necrosis only at the tip (Figure 7 B). The third leaf of the wild-type was entirely rolled and almost half of the leaf was necrotic, starting from the tip (Figure 7C). In contrast third leaves of hebiba and cpm2 were necrotic only at the tip, and approximately 2/3 of the leaf remained unfolded. Hence the mutants deficient in the phytohormone jasmonate showed a less salt sensitive phenotype under the conditions applied.

3.2 Effect of salt stress on root elongation

Roots are the first line of defense against salinity stress, and its growth would be a clear reflection to the adaptive response to salt stress. In order to compare the effect of salinity on growth in wild type and mutants we examined the length of seminal roots after 7 days in complete darkness. Roots of the mutants hebiba and cpm2 were shorter under these conditions when grown in absence of supplemented NaCl or at concentrations below 12mM. However they developed considerably longer primary roots than the wild type at high salt concentrations (62.5 and 125 mM). At 62.5 mM cpm2 and hebiba roots length were 40.14 mm and 47.54 mm, respectively, while WT was of 25.51 mm. At high salinity stress (125 mM) the corresponding values were 10.70 mm and 8.89 mm for cpm2 and hebiba, respectively, compared to 3.12 mm in case of WT. Neither wild type nor JA-biosynthesis mutants were able to germinate on medium containing 250mM NaCl (Figure 8 A and B).

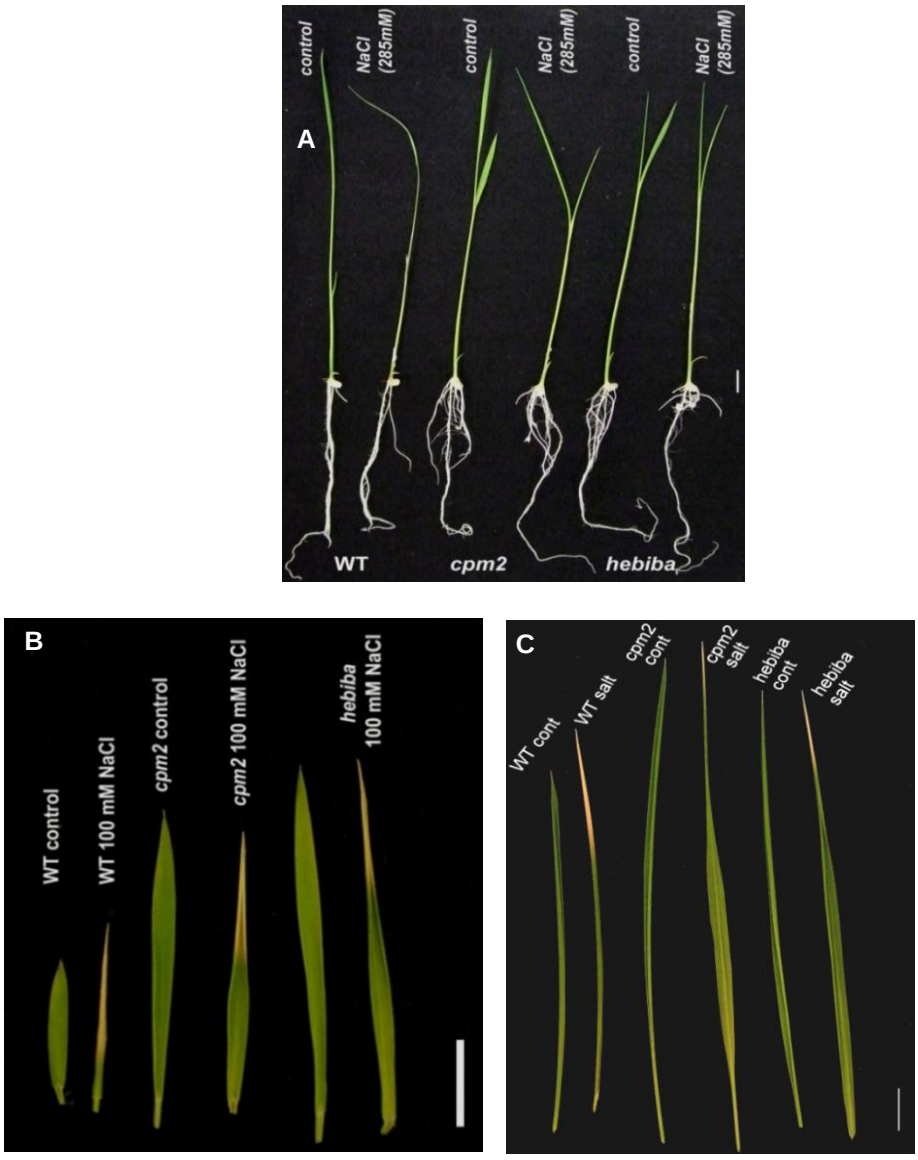

Figure7. JA-mutants lines showed a less sensitive to salt stress phenotype.10 days old rice seedlings were allowed to be stressed by salt stress in two different strategies, short term (285mM for 24hrs) and long-term (100mM for 72 hrs). The phenotype appeared from the damage of salinity was illustrated. Bar equal to 10mm. A) Short-term salt stress (285mM for 24hrs). B) Long-term salt stress effect on the second leaf. C) Long-term salt stress effect on the third leaf.

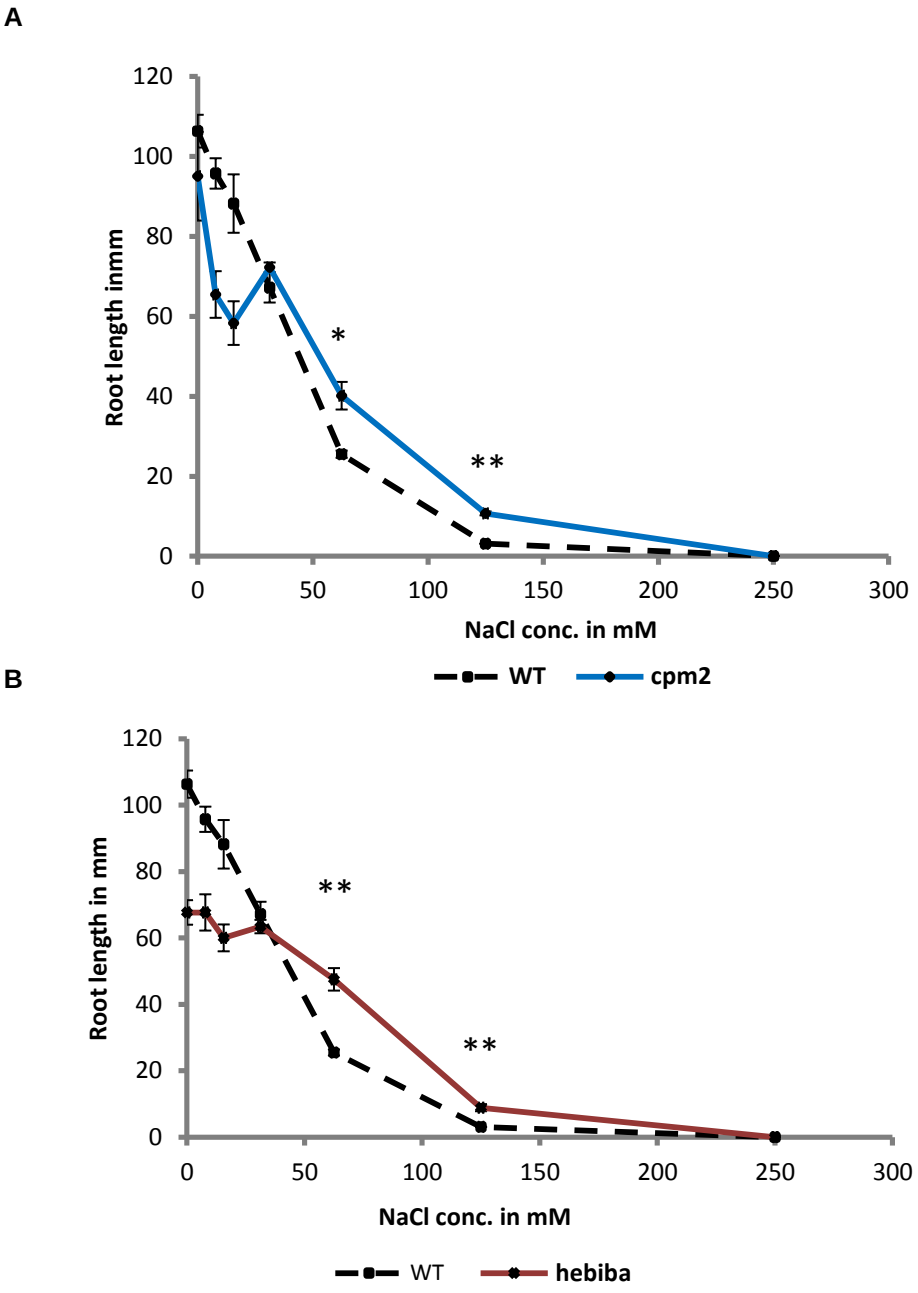

Figure 8. Jasmonte mutants' roots showed enhanced root growth comparing to the wild-type under salinity stress. The seeds of both WT and JA-mutants were germinated in solid phytoagar medium containing different concentrations of NaCl, the grown roots length were scanned and measured using Image J software. A) The comparison between WT and JA-mutant (*cpm2*) on the level of root length. B) The comparison between WT and JA-mutant (*hebiba*) on the level of root length. Values represent the mean of at least three independent experiments +\- SE. Means followed by one star and two stars among treatments are significantly different, according to student T-test ($P<0.05$ and $P<0.01$, respectively).

3.3 JA- mutants accumulated less Na^+ ions in shoots but not in roots

As was observed in Figures 7 and 8 the mutants showed less salt stress damage phenotype, so it is worth to test how the absence of JA in the mutants could affect on Na+ ions influxing. Therefore, we examined the content of sodium ions in roots and shoots of after 3 days in 100 mM aqueous solution of NaCl. The shoots of the mutants accumulated less sodium ions compared to the wild type (62.55 mg/gDW (cpm2) and 55.5mg/gDW (hebiba) to 84.93 mg/gDW ((wild type), respectively). However, there was no significant difference between the mutants and the wild type in sodium contents in roots (Figure 9). Hence, weaker stress symptoms observed in mutant leaves may partially be due to a lower level of sodium ions in the tissue.

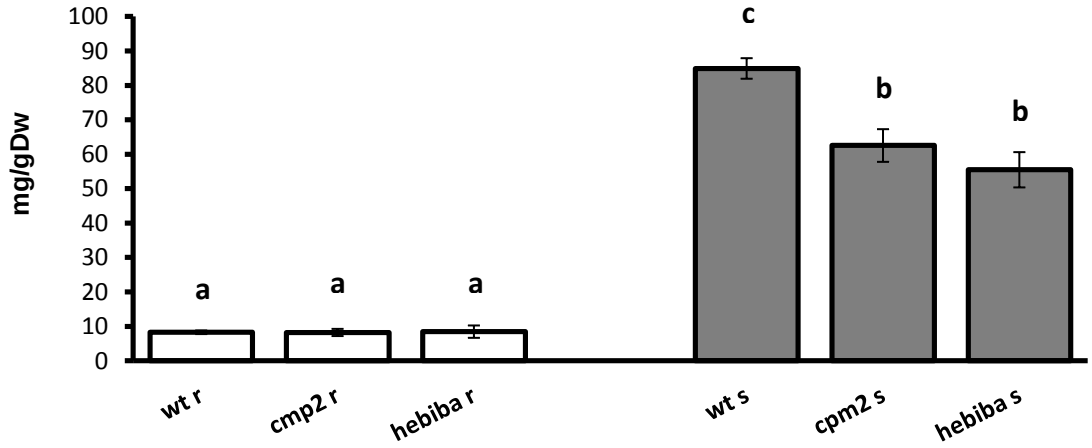

Figure 9. 10 days old rice seedling representing WT and JA-mutants were stressed in aqueous NaCl 100mM solution for three days. The blank bars representing sodium ion content in roots, while gray bars for shoots sodium ions contents. Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

3.4 Effect of salinity on the chlorophyll contents parameters

To research the effect of salinity on the performance of photosynthetic machinery in tested rice plants, the chlorophyll contents parameters were estimated in the leaves of wild type and JA-mutants lines. The level of chlorophyll in the leaves was shown in Figure 10 A, the wild type leaves under salt stress showed a significant reduction in the contents of total chlorophyll comparing to the control conditions (0.54 to 1.58 mg Chl/g fresh Wt, respectively). On the other hand, the salinity stress that is triggered by NaCl

did not affect on the level of total chlorophyll in JA-mutants, as the difference between control and salt stressed conditions was not statistically significant. It has been found that there is no difference between the wild type and JA-mutants in terms of total chlorophyll content under salinity stress. Figure 10B showed that the salinity stress caused a significant reduction in the content of chlorophyll a with all treatments; nevertheless, there is no difference within the wild type and the mutants under salinity stress. The level of chlorophyll b was also observed to be reduced under salt stress comparing to the control conditions in case of wild type only, while JA-mutants showed a relatively stable Chl b level (Figure 10C) .

3.5 Oxidative stress manipulation in JA-mutants

3.5.1 Enhanced mitigation of oxidative damage in JA-mutants

As an attempt to study the toxic effect of overproduced ROS under salt stress on tested plants, firstly we have examined the level of lipid peroxidation, as a biomarker for the toxic effect of ROS on the plasma membrane of the living cells. Lipid peroxidation was estimated by measuring malondialdehyde (MDA), a molecule that is generated during the degradation of biological membranes by reactive oxygen species (ROS). Generally, the level of MDA was increased in both wild type and JA-mutants (only hebiba) in response to salt stress. Nevertheless, the level of MDA was higher in wild type shoots (10.97 µmol MDA g/FW) compared to JA-biosynthesis mutants (7.06 µmol MDA /gFW (cpm2) and 5.80 µmol MDA /gFW (hebiba), respectively) under salinity stress (Figure 11A). Since we found evidence that less ROS might be present less in mutant shoots we determined levels of H_2O_2 in plant tissues. The wild type accumulated 84.1 µmol gFW^{-1} hydrogen peroxide while the mutants contained significantly less H_2O_2 (50.58 and 50.43 µmol /gFW , for cpm2 and hebiba, respectively, Figure 11B). Unlike the mutants, the WT seedlings leaves significantly accumulated more H_2O_2 under salt stress. We observed that the level of H_2O_2 in hebiba was lower than WT and cpm2 under control conditions.

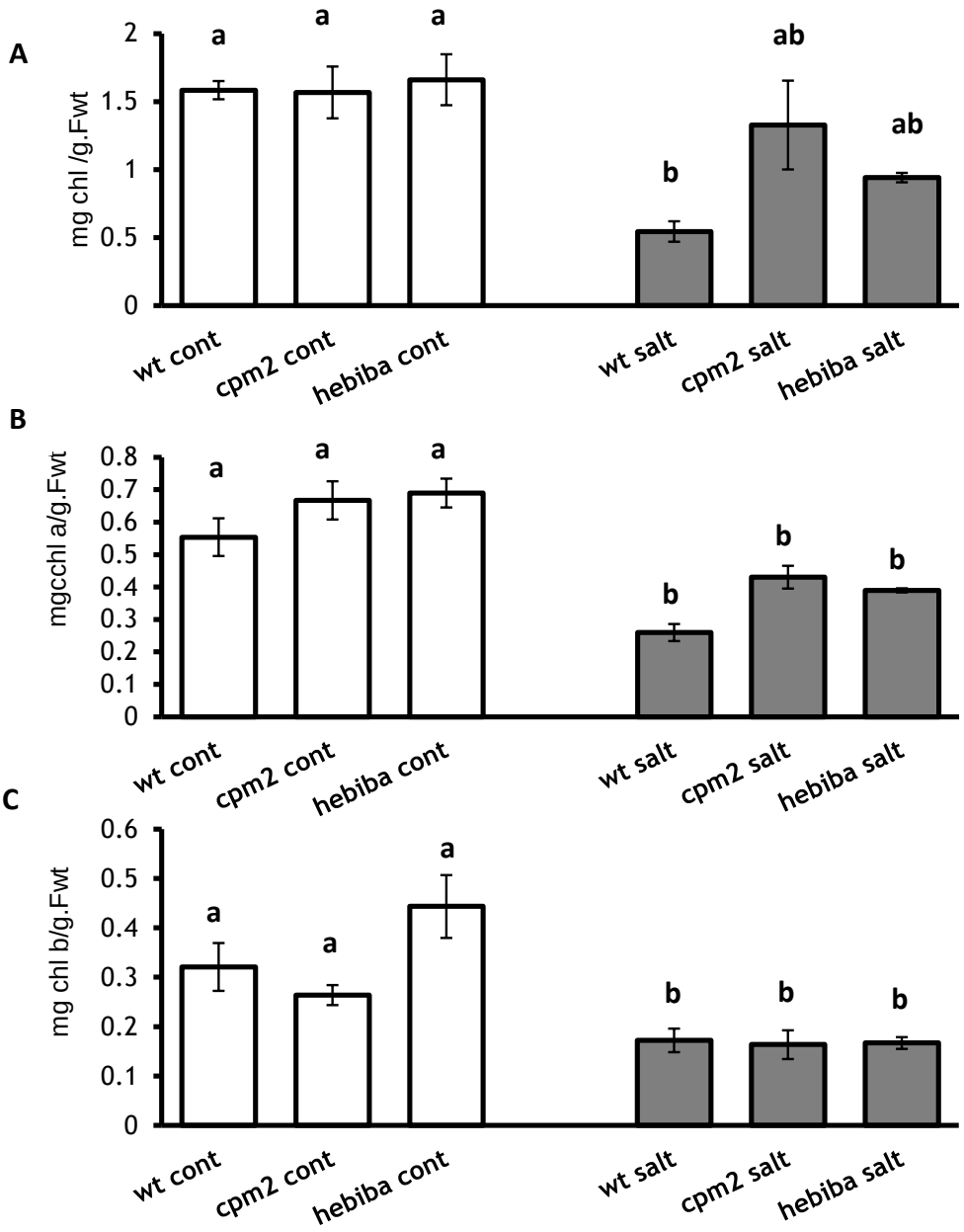

Figure 10. The chlorophyll contents of the mutants did not strongly affected by salinity comparing to the wild type in which the total chlorophyll and chlorophyll b were reduced significantly. A) Total content of chlorophyll, B) chlorophyll a content, and C) chlorophyll b content. Blank bears representing the control (water), gray bare representing the salt stressed leaves. Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

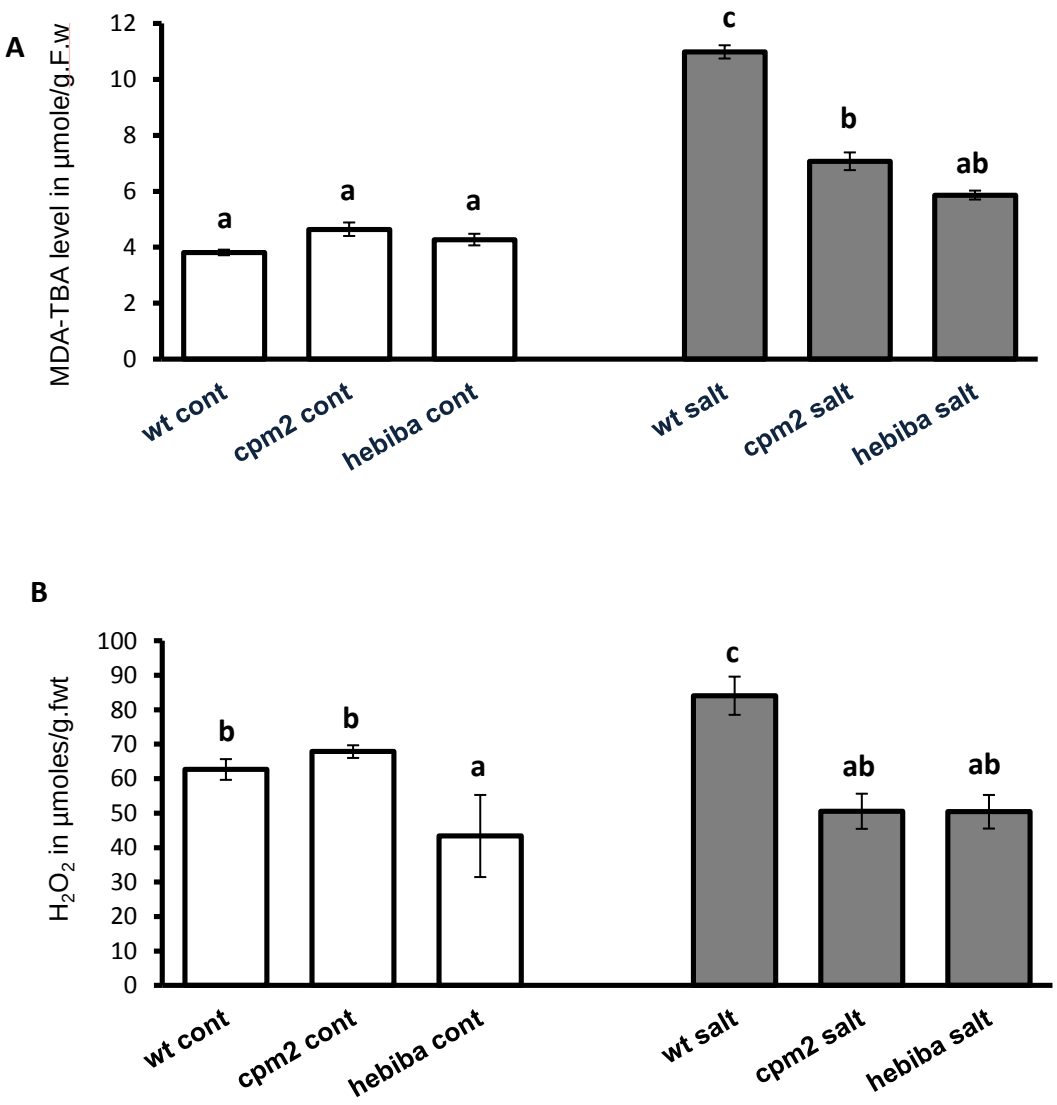

Figure11. Reduced lipidperoxodation and hydrogen peroxide levels were found to be lower in the JA-mutants comparing to the WT under salinity stress.A) Level of MDA (malondialdehyde) was estimated in WT and JA-mutants shoots in control (blank bars) and salt stressed (gray bars).B) Aqueous peroxide in control (blank bars) and salt stressed shoots (gray bars) in terms of hydrogen peroxide depending on a pre-prepared standard curve with different concentration of H_2O_2. Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

3.5.2 Evaluation of antioxidants amounts

We have also estimated the level of soluble proline as a sign of responding to abiotic stress. The control samples in both WT and the mutants did not contain a detectable amount of soluble proline (Figure 12A). In all the genotypes tested exposure to salinity lead to a sharp increase in proline content. However, the estimated soluble proline amount in the mutants' shoots was significantly lower than wild type (0.4 and 0.28 to 1.53 µmol gFW^{-1} in cpm2, hebiba, and wild type, respectively).

Total polyphenolic acid content (TPC) and total flavonoids content (TFC) were estimated in the leaves of wild type and JA-mutants in both control and salt stressed conditions. In case of TPC (Figure 12B), both wild type and JA-mutants showed a reduction in the level of TPC in responding to salinity stress (from 7.8 to 4.21, 7.08 to 4 and 7.5 to 3.94 mg GA eq./g.Dw, for wild type, cpm2 and hebiba respectively). Nevertheless, this method could not show a difference within the treatments under salt stress. In TFC content (Figure 12C), we observed a reduction in the content of TFC in rice leaves with all treatments, from 47.19 to 22.37, 44.62 to 22.10 and 46.9 to 23.94, in case of wild type, cpm2 and hebiba, respectively. Also, we did not find a significant difference within the wild type and the mutants under salt stress.

3.5.3 Non-enzymatic antioxidants ability JA-mutants under salt stress

3.5.3.1 DPPH scavenging activity was higher in JA-mutants

In order to test the ability of the plants to detoxify the ROSs, we estimated the total antioxidants ability of the methanolic extract to scavenge the stable radical DPPH. The idea of this test is to test how the methanolic extract of rice leaves will successfully scavenge the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and we check this scavenging activity spectrophotometrically at 515nm. As shown if Figure 13A, the methanolic extract of all three genotypes succeeded to scavenge the stable radical DPPH in a dose dependant manner. Based on the IC_{50}% values (Figure 13B) out of the extracts tested the one obtained from hebiba was most efficient in scavenging ROS (221.99 µg ml^{-1}), followed by cpm2 (236.95 µg ml^{-1}) and wild type (317.79 µg ml^{-1}). As a positive control butylated hydroxyanisole had on IC_{50}% value of 8.23 µg ml^{-1}. The more

the lower IC_{50}% value, the more the antioxidant ability. The JA-biosynthesis mutants accumulate less MDA which might be due to their ability to detoxify salinity stress related ROS more efficiently than their wild type background.

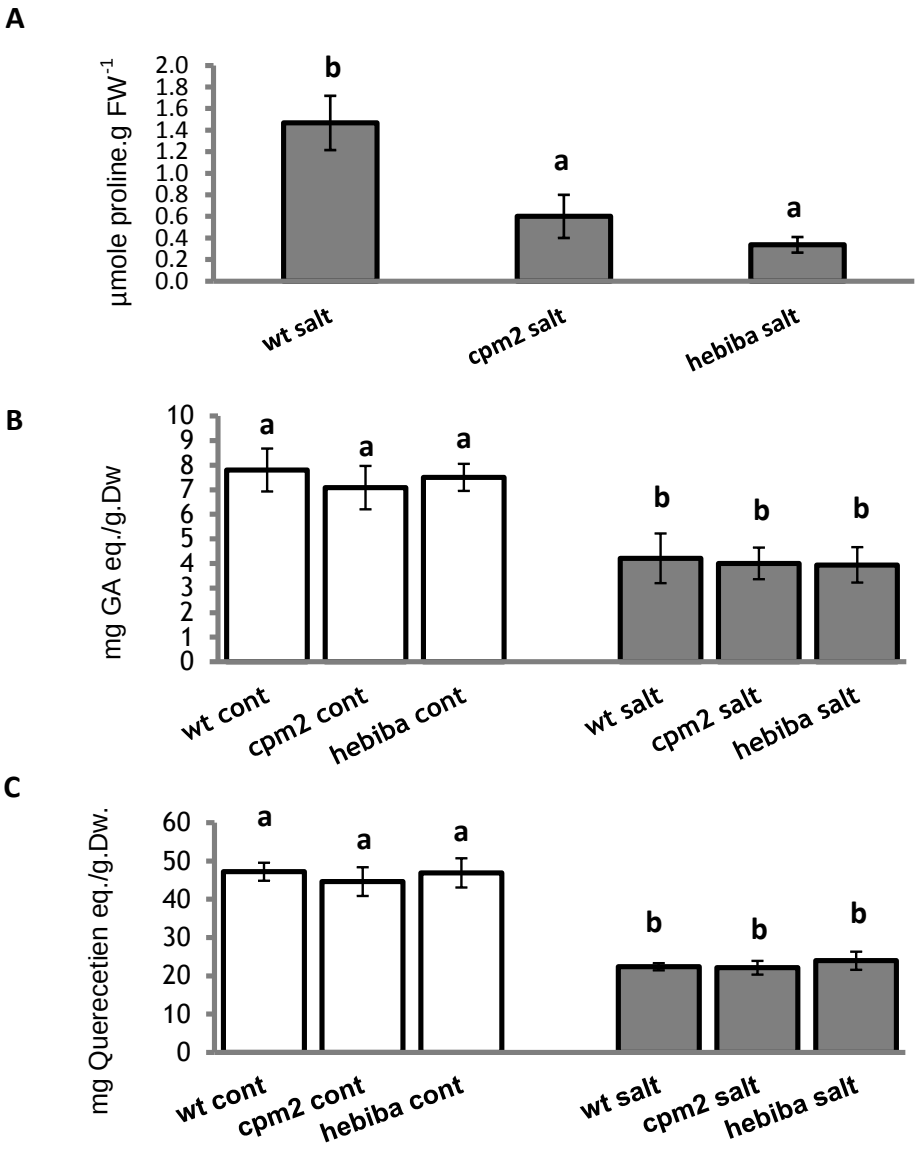

Figure 12. The level of soluble proline under salt stress was lower in JA-mutants comparing to wild type. Both TPC and TFC was reduced in response to salt stress, however, there levels were not different within the treatments under salt stress. A) Soluble proline in WT and JA-mutants under salt stress, control not detectable. B) Level of total polyphenolics contents (TPC) in WT and JA-mutants shoots in control (blank bars) and salt stressed (gray bars). C) Level of total flavonoids contents in WT and JA-mutants shoots in control (blank bars) and salt stressed (gray bars). Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

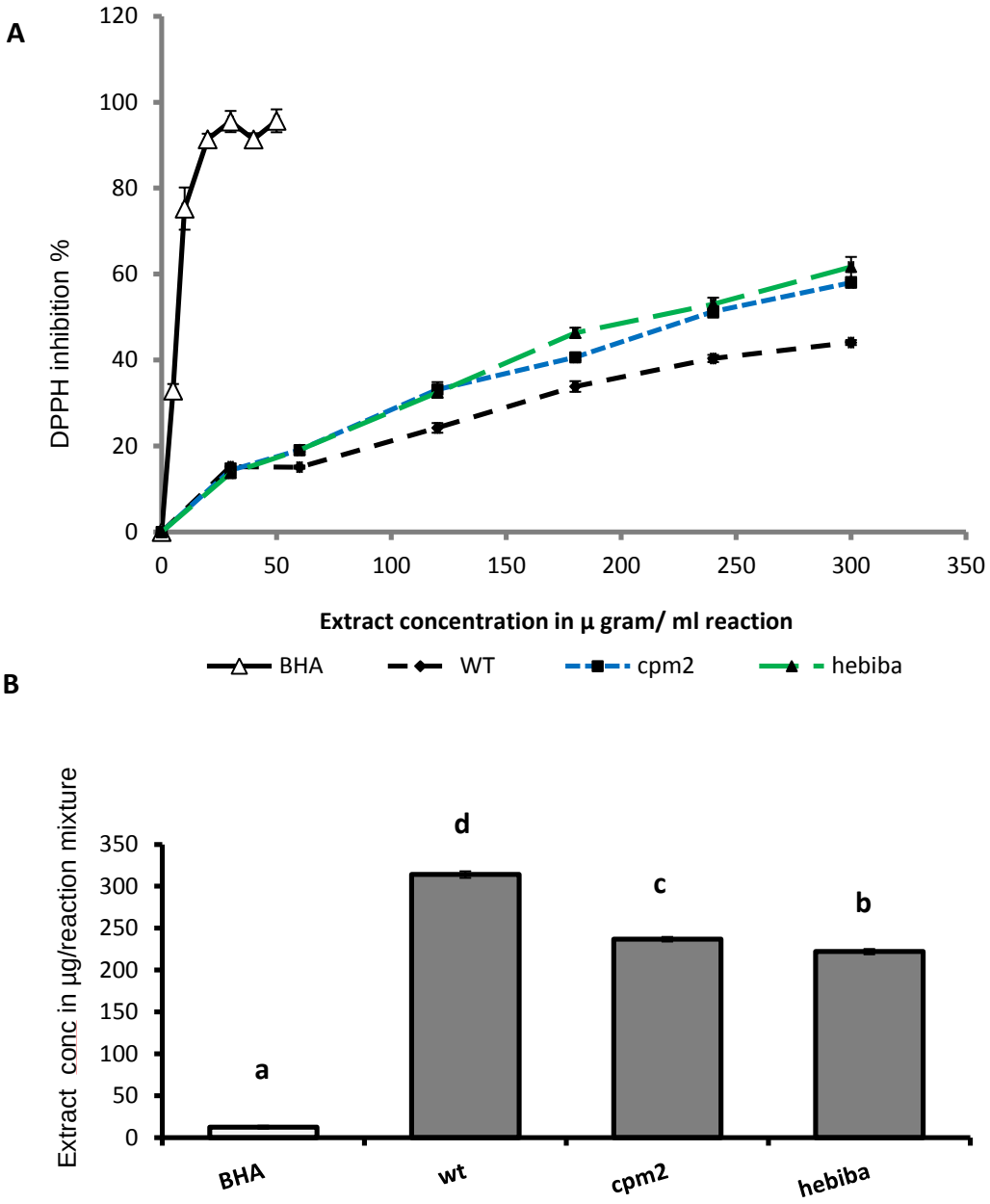

Figure 13. The methanolic extract scavenging activity of the free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was higher in the JA-mutants more than WT under salt stress. A) DPPH free radical scvaenging activity of standard Butylated hydroxyanisole (BHA) and methanolic extract of wild-type and JA-mutants under salinity stress of 100mM NaCl for 3 days. B) The values of IC_{50}% was calculated fron figure A depending of regression analysis.). Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

3.5.3.2 JA-mutants detoxify superoxide anion better than WT

As previously mentioned in the introduction, the plants over accumulated many types of ROS under stressed conditions, as salinity. Superoxide radical anion $O_2^{\cdot -}$ is the first born type of free radicals, therefore, the ability of the antioxidant machinery in the plant should be very concerning about removing or scavenging this harmful excited molecule before damaging many important biomolecules as DNA and metabolic or photosynthetic enzymes.

We have tested the non-enzymatic antioxidants ability to detoxify superoxide anion specifically. We have used SOSA (superoxide scavenging assay) test. Superoxide radicals are generated in riboflavin, methionine, illuminate and assayed by the reduction of nitroblue tetrazolium (NBT) to form blue formazan ($NBT2^+$). As shown in figure 14 A, B and C, the aqueous extract of rice leaves of both wild type and JA-mutants scavenge the in-vitro produced superoxide anions in a dose dependent manner in both control and salt stress conditions. Also we observed that the inhibition percentage as a marker of scavenging activity is dramatically reduced under salt stress conditions comparing to the control conditions in all treatments in SOSA test. Depending on the data from the inhibition percentage of each extract, we calculated the IC_{50}% of each case and presented it in Figure 14 D. we observed that IC_{50}% values of all treatments were reduced under salt stress in relative to the control conditions. Interestingly, we observed that the IC_{50}% values for the JA-mutants were much significantly lower than wild type under salt stress, however, there is no difference in case of control conditions. It was 359.74 and 293.89 to 437.83 in case of cpm2 and hebiba comparing to wild type, respectively.

3.5.3.3 Scavenging activity of hydrogen peroxide

We have measured the non-enzymatically antioxidants ability to detoxify hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH.-) anions. The produced anions of superoxide radicals will be dismutated enzymatically through the enzyme superoxide dismutase (SOD) to hydrogen peroxide. In spite that hydrogen peroxide is a stable molecule and not a free radical like superoxide anions; it has damage oxidative effects

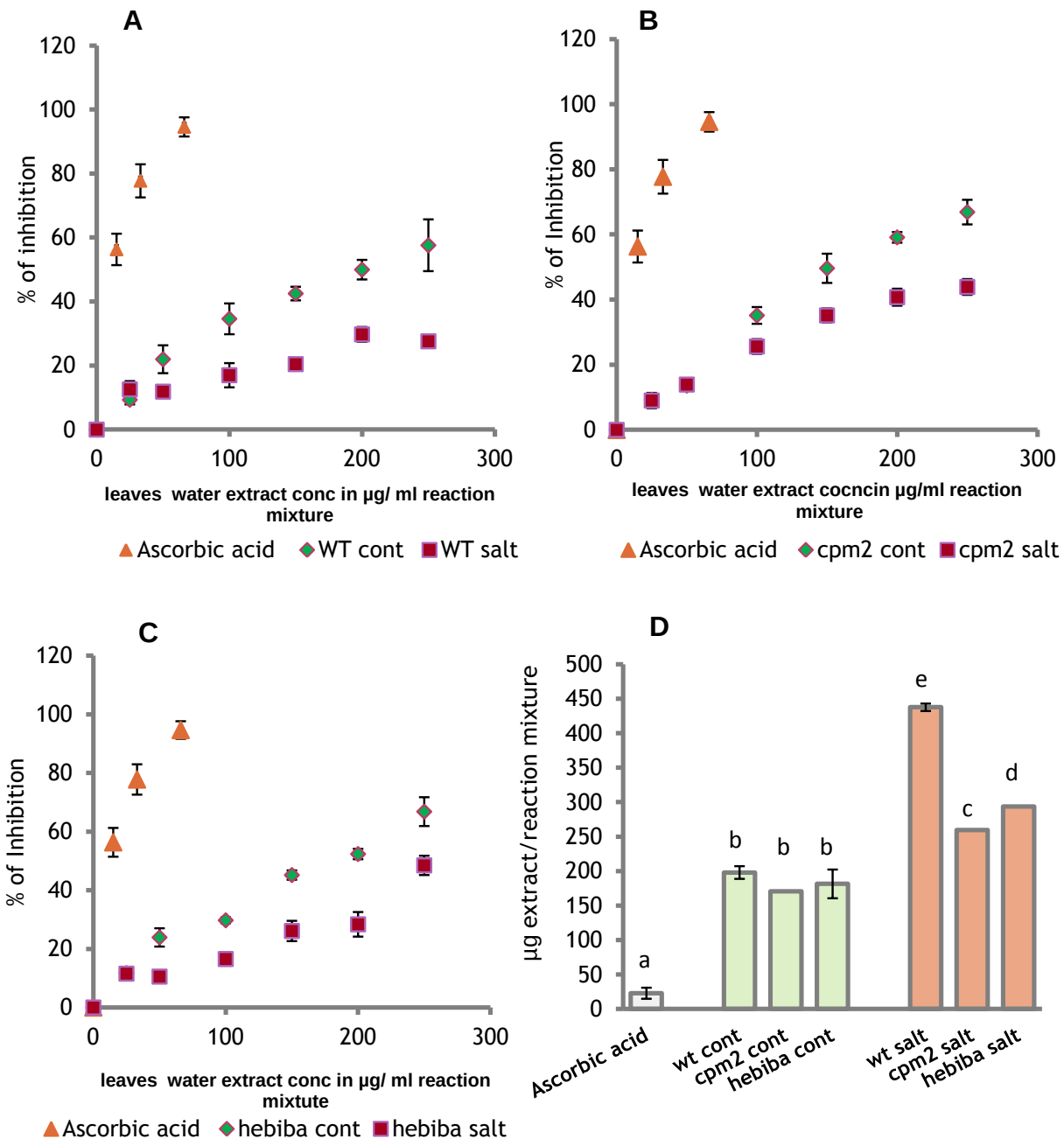

Figure 14. The superoxide scavenging activity (SOSA) was detected successfully in a dose dependent manner, while the IC_{50}% values of JA-mutants were lower than wild type indicating higher antioxidants ability. SOSA test of aqueous extracts of wild-type (A), *cpm2* (B) and *hebiba* (C) under control and salinity stress of 100mM NaCl for 3 days. D) The values of IC50% of WT, *cpm2* and *hebiba* under control and salt stress conditions, the standard ascorbic acid was used as internal positive control. Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

on the plasma membrane and many enzymes. The non-enzymatic scavenging ability of the rice leaves under control and salinity stressed was measured biochemically in its aqueous extract. As illustrated in Figure 15 A, B and C, the method we have used in measuring the scavenging activity of H_2O_2 did not show that there was no ability to scavenge the in vitro produced H_2O_2 in both control and salt stress condition with all treatments. Nevertheless, we tested the viability of the test by proofing the scavenging activity of BHA as a well known H_2O_2 scavenger. Figure 15 D showing that there is no difference between the value of IC_{50}% in both wild type and JA-mutants in control and salt stress conditions.

3.5.3.4 Hydroxyl radical scavenging activity in both WT and JA-mutants

The hydroxyl radicals are produced from hydrogen peroxide non-enzymatically, however, the hydroxyl radicals are very active and has the strongest and fastest oxidative damage on the cell membranes and biomolecules. The ability of the aqueous extract of rice leaves to detoxify the in vitro produced hydroxyl radicals of both wild type and JA-mutants under control and salt stress conditions was tested.

As shown in Figure 16 A, B and C that the aqueous extract of rice leaves in all treatments succeeded in detoxifying the hydroxyl radicals in a dose dependent manner. The IC50% values of the aqueous extracts of wild type and JA-mutants leaves had no significantly differences under salt stress, as shown in Figure 16 D.

3.5.4 Effect of salinity on the antioxidants enzyme profile

We have measured the activity of six different antioxidants enzymes as an attempt to profile the enzymatic antioxidants mechanism which plants use in addition to the non-enzymatic strategy to manage ROS levels under control and stressed conditions. These enzymes are: Superoxide dismutase (SOD), Catalase (CAT), Ascorbate peroxidase (APX), Peroxidases (POD), Glutathione Reductase (GR), and Glutathione-S-transferase (GST). The protein contents were measured using Bradford assay. The enzyme superoxide dismutase (SOD) is the first line of defense against the produced superoxide anions in order to dismutate it to hydrogen peroxide.

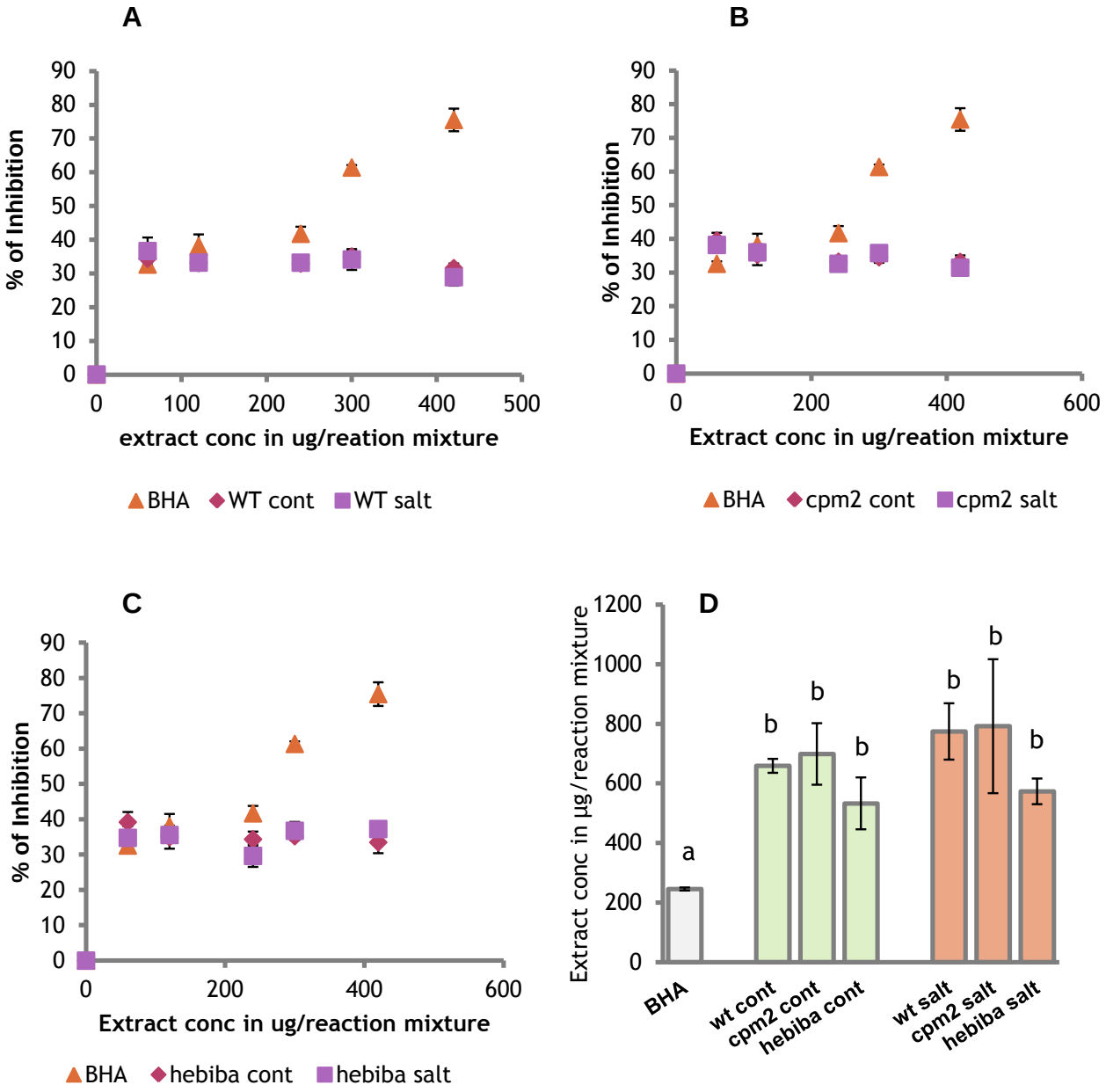

Figure 15. The hydrogen peroxide scavenging activity (HPSA) was not detected successfully in a dose dependent manner, while the IC_{50}% values of all treatments had no significant difference. HPSA test of aqueous extracts of wild-type (A), *cpm2* (B) and *hebiba* (C) under control and salinity stress of 100mM NaCl for 3 days. D) The values of IC50% of WT, cpm2 and hebiba under control and salt stress conditions, the standard BHA was used as internal positive control. Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

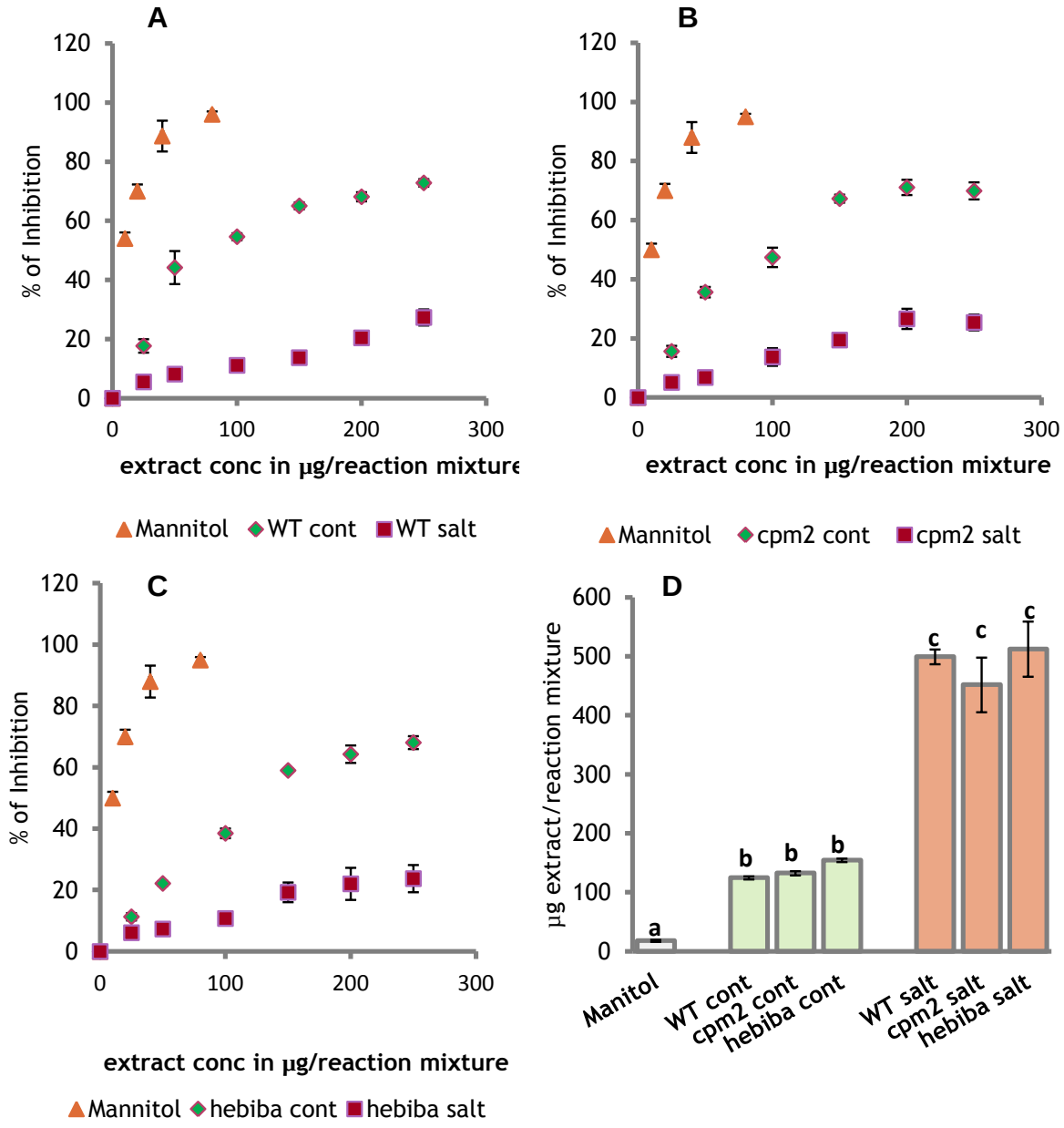

Figure 16. The hydroxyl radical scavenging activity (HRSA) was detected successfully in a dose dependent manner, while the IC_{50}% values of wild type and JA-mutants had no significant difference. HRSA test of aqueous extracts of wild-type (A), *cpm2* (B) and *hebiba* (C) under control and salinity stress of 100mM NaCl for 3 days. D) The values of IC50% of WT, cpm2 and hebiba under control and salt stress conditions, the standard BHA was used as internal positive control. Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

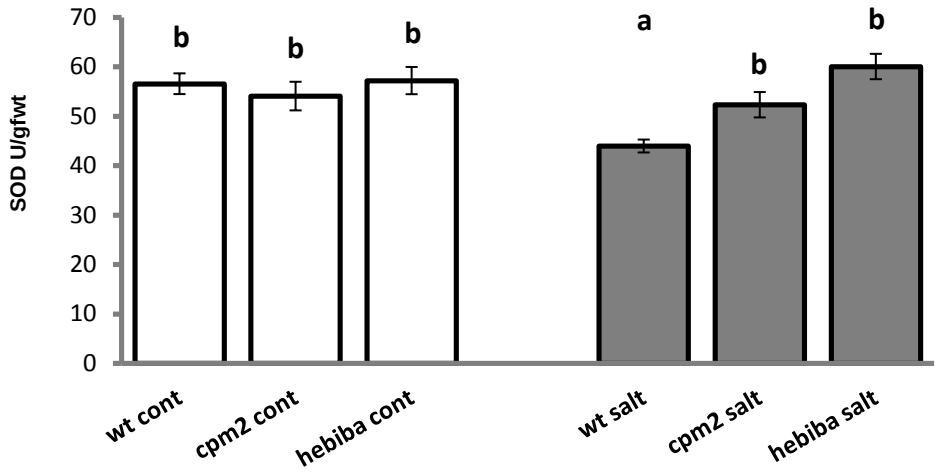

Figure 17: The activity of Superoxide dismutase (SOD) was found to be more stable in JA-mutants under salt stress comparing to the wild type. Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

As shown in figure 17, the activity of SOD was reduced significantly in wild type leaves in response to salt stress (from 56.53 to 43.93 U/gFwt). On the other hands, the JA-mutants SOD activity under salt stress was stable and did not significantly changed in relative to control conditions, from 45.03 to 52.28 and from 57.17 to 60.01 in both cpm2 and hebiba, respectively. The activity of catalse enzyme was measured in control and salt stressed conditions. In all treatments the activity of catalse was significantly reduced in response to salinity stress comparing to control conditions. In case of wild type it was reduced from 1.6 to 1.0 U/mg protein/min, in cpm2 it was reduced from 1.1 to 0.8 U/mg protein/min and in case of hebiba the activity of catalse was reduced from 1.4 to 0.7 U/mg protein/min. on the other hand we could not find a difference within salt stress between wild type and JA-mutants (Figure 18 A).

Figure 18 B illustrated our investigation of the activity of ascorbate peroxidase (APX) was also determined, the wild type showed an increase in the activity of APX under salt stress (19.45 to 61 U/mg protein/min), however, JA-mutants (cpm2 and hebiba) showed

a significant reduction in APX activity comparing to wild type (61, 28.15 and 33 U/mg protein/min, respectively). The APX activity in both wild type and JA-mutants had no significantly differences under control conditions.

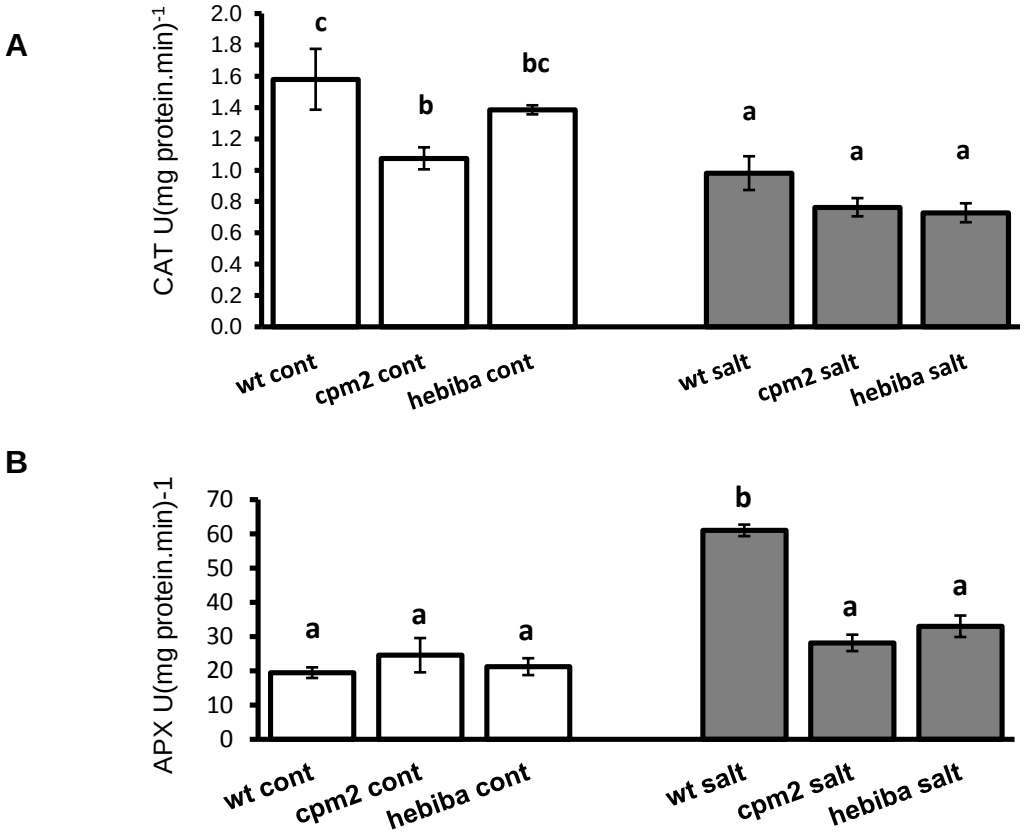

Figure 18. The activity of catalase (CAT) was found to be reduced in all treatments, while ascorbate peroxidise (APX) activity was found to be less in JA-mutants under salt stress comparing to the wild type. A) CAT activity. B) APX activity.Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

The activity of peroxidases enzymes was estimated in the leaves as shown in Figure 19. The JA-mutants has a higher activity of peroxidases enzymes comparing to wild types in response to salt stress. In case of wild type, the POD activity in the control was 141.48, while under salt stress was 140.25 U/mg protein/min, indicating no significant difference. On the other hand, cpm2 showed an elevation in POD activity from 92.6 to be 172 U/mg protein/min in control and salt stress conditions, respectively. In hebiba

POD activity also significantly increased from 111.68 in control to 168.88 U/mg protein/min.

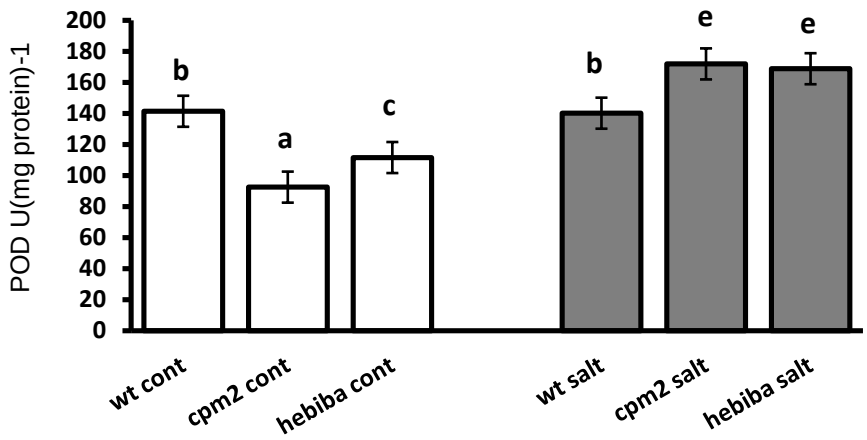

Figure 19: The activity of peroxidises was found to be higher in JA-mutants in relative to wild type. Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

The activity of Glutathion Reductase (GR) was measured depending on the observation of NADPH oxidation by oxidized glutathione (GSSG) which leads to a reduction in the absorbance at 340nm. As shown in Figure 20A, the GR activity for wild type and JA-mutants under control conditions was statistically similar. However, in response to salinity stress, hebiba leaves only showed an increase in GR activity (25.6 U/mg protein/min) to be higher than wild type (16.82 U/mg protein/min) and cpm2 (19.95 U/mg protein/min) under salt stress.

We also measured the activity of Glutathion-S-transferase (GST) in both wild type and JA-mutants leaves protein extract under control and salt stress conditions. GST activity was estimated by observing the increase of the absorbance at 340 nm as a result of the formation of GS-DNB (Glutathion dinitrobenzene). As shown in Figure 20 B, the JA-mutants (cpm2 and hebiba) has higher GST activity under salt stress in relative to wild type, 281.33, 267.09 and 176.05 U/mg protein/min, respectively. Additionally, we observed that GST activity in case of cpm2 under control conditions was lower than wild type and hebiba .

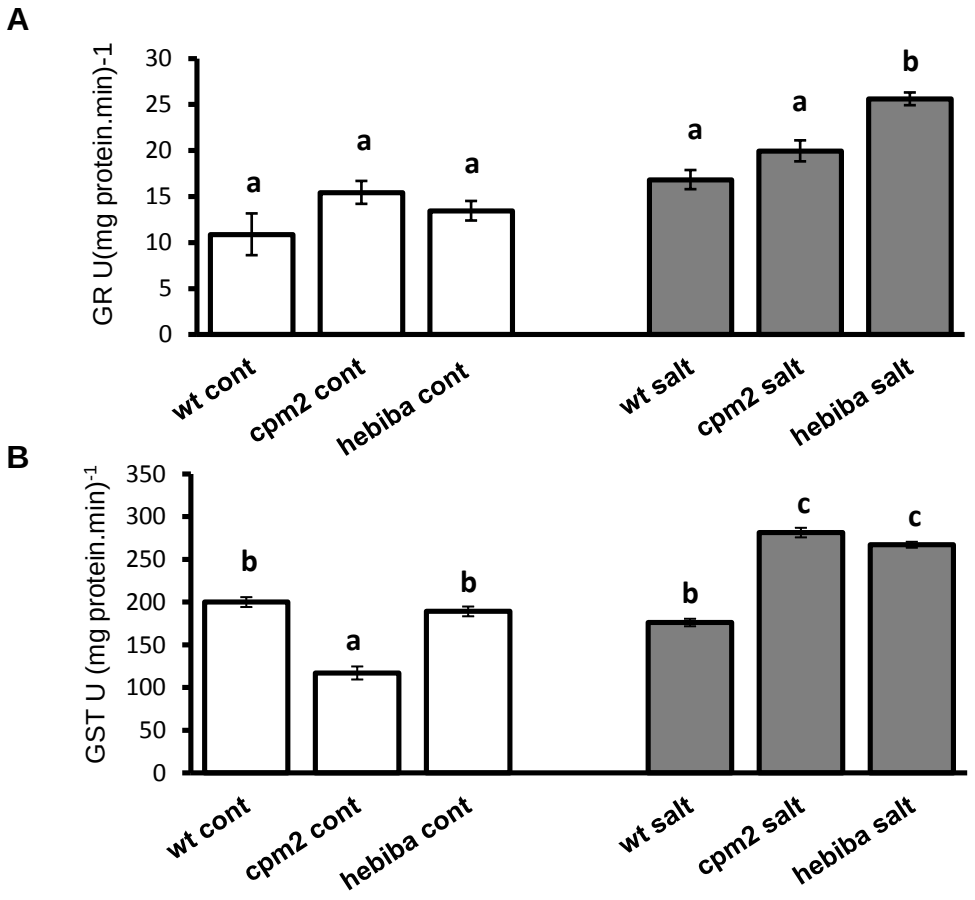

Figure 20. The activity of Glutathione reductase (GR) and Glutathion-S-transferase was found to be higher in JA-mutants in relative to wild type. A) Glutathion Reductase activity. B) Glutathion-S-transferase (GST). Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

3.6 JA-deficiency affects gene expression in response to salt stress

Since we found that JA-biosynthesis mutants show differences in the response to salinity on the physiological and biochemical level, we were interested to investigate the consequences of JA-deficiency on the regulation of selected genes in response to salt stress.OsNHX1 (encoding vacuolar Na^+/H^+ vacuolar antiporter) has an important role in cellular pH and Na^+ homeostasis by scavenging the sodium ions from the cytoplasm into the vacuoles which considered to be very important strategy to protect the enzyme system in the cytoplasm and to maintain low Na^+/K^+ ratio. In both, the mutants and the wild type, the gene was induced by salinity (Figure 21a). However, the relative change in expression of OsNHX1 compared to control plants exposed to H_2O was found to be significantly lower in the mutants compared to the wild type at two time-points investigated (24 h: 9.6-, 6.0- and 3.1-fold, and 72 h: 18.5-, 10.7- and 11.1-fold for wild type, cpm2 and hebiba, respectively).

Next, we studied the transcriptional regulation of the gene encoding a protein similar to the enzyme serine acetyltransferase which is a rate limiting enzyme in the synthesis of sulfur containing amino acid cystein which is very important in the production of some antioxidants molecules as glutathione. We refer to this gene as OsSAT (AK287779). Different from OsNHX1 this gene was only slightly induced after 24 h, but showed an almost 7-fold increase in its transcripts 72 h (Figure 21B). In contrast OsSAT was not induced in cpm2 and hebiba. In order to investigate the role of JA signaling in modulating salinity stress response, we tested the transcriptional regulation of OsJAZ13 (encoding Jasmonate ZIM domain protein 13) which belongs to a family of plants specific genes encoding proteins characterized by a conserved Jas domain (Jasmonate associated), which is important for the repression of jasmonate signalling. The expression of OsJAZ13 was induced by salt stress, however, the relative change of transcripts was found to be significantly smaller in the mutants at both time points examined (Figure 21C). Nevertheless, the JA-biosynthesis mutants showed an induction of this gene, especially after 72 h of stress treatment (6.4-fold induction in cpm2, 12.5-fold in hebiba).

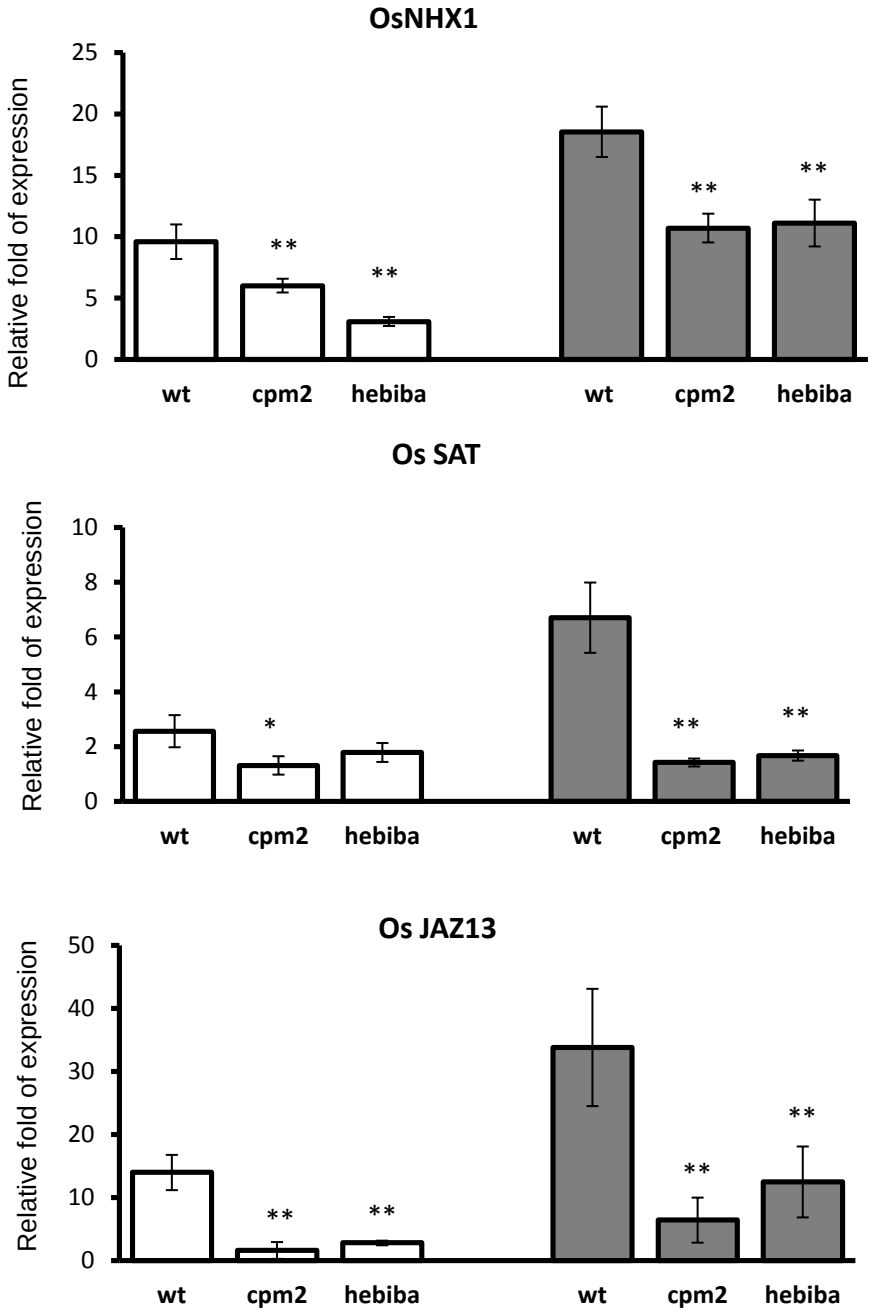

Figure 21. The expression profile of some salt stress related genes under salt stress using aqueous NaCl solution (100mM) in two time points , 24 hrs (Balnk bars) and 72 hrs(Gray bars). Values represent the mean of at least three independent experiments +\- SE.

For examining effects on the pathway for the production of hydrogen peroxide, OsOXO.4 gene expression was tested. This gene encodes one of the enzymes that are responsible for the production of hydrogen peroxide under biotic and abiotic stress, namely oxalic acid oxidase. OsOXO.4 expression was strongly induced under salinity stress (Figure 22 A). Wild type shoots showed much higher relative changes in the amount of transcripts compared to the mutants (72 h: 267.7-, 88.6-, and 55.5-fold increase in wild type, cpm2 and hebiba, respectively). Before 72 h and in absence of salt OsOXO.4 was almost not expressed.

The expression of OsNCED5 encoding for 9-cis-epoxycarotenoid dioxygenase, a key enzyme in ABA synthesis, was also investigated. Generally, OsNCED5 gene transcription was found to be induced by salinity stress. The transcripts were found to be stronger induced in JA-biosynthesis mutants compared to the wild type (Figure 22 B). The induction factors after 24 h were 2.5, 5.0 and 3.6 for wild type, cpm2 and hebiba, respectively, and after 72 h they were 2.3, 9.0 and 5.5 for wild type, cpm2 and hebiba, respectively. We also checked the expression of OsNR1 (Os02t0770800-1), a gene encoding for Nitrate Reductase (NR), a key enzyme in the synthesis of nitric oxide (NO) in plants. The induction of OsNR1 after 24 h was higher in the mutants (27.2 and 12.0 for cpm2 and hebiba, respectively), compared to the WT (5.1) (Figure 22 C).

Taken together we found that several salt stress responsive genes are differentially expressed in mutant and wild type plants. Genes which are involved in the response of plant metabolism to salt stress, such as OsSAT and OsOXO.4 were not or much less responsive to salt stress, while genes involved in stress protection such as the ABA-biosynthesis gene OsNCED5 or the NO biosynthesis gene OsNR1 were more responsive in the mutants.

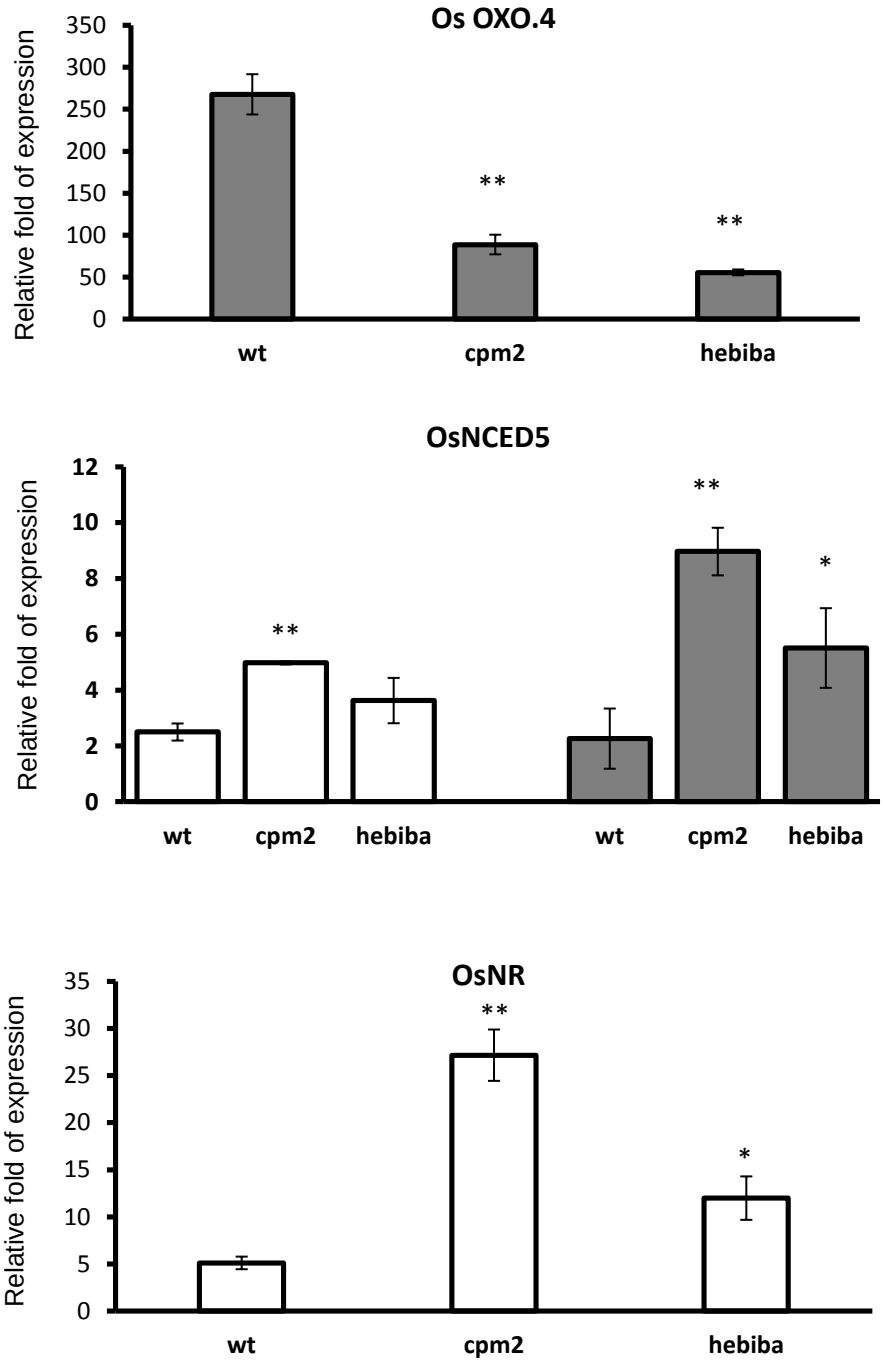

Figure 22. The expression profile of some salt stress related genes under salt stress using aqueous NaCl solution (100mM) in two time points , 24 hrs (Blank bars) and 72 hrs(Gray bars) . Values represent the mean of at least three independent experiments +\- SE.

3.7 Effect of salinity on level of some plant hormones in JA-mutants

In order to elucidate the role of plant hormones for mediating salt adaptive stress in JA-mutants and to translate the done gene expression profile to physiology, we have measured the level of some plant hormones (Jasmonic acid, Jasmonate isoleucine and Absisic acid) in response to salt stress in both wild type and JA-biosynthesis mutants lines. Also, we have measured the level of OPDA (oxi-phytodecatrenoic acid) which is the only JA biosynthesis precursor that has a signalling effect. Additionally, and equally important the level of ABA, as well known hormone in abiotic stress, was also measured to find out its cross talk with JA. 10 days old seedlings were subjected to salt stress of 100 mM NaCl then the leaves were harvested after 1 and 6 hrs and the fresh weight was detected then leaves were allowed to be flash frozen in liquid nitrogen and kept in -80Cº.

3.7.1 WT accumulate more Jasmonates after sensing salt stress

For the level of jasmonic acid (JA) we have found that its level increased significantly in WT in response to salt stress after 1 and 6 hours, 145.33 and 200 pmol/gFwt (Figure 22 A). However, the level of JA under salt stress was significantly stable and did not change in both time points. On the other hand, the level of JA was observed to be not changed under salinity stress in relative to control conditions in time points, 1 and 6 hrs. The level of JA-Ile or jasmonate isoleucine, the biologically active form of jasmonic acid was also measured in both WT and JA-biosysnthsis in control and salt stress conditions. As shown in Figure 22 B, the level of JA-Ile was found to be elevated significantly in WT leaves under salt stress after 1 hr (155.33 pmole/gFwt) and 6 hrs (203.33 pmole/gFwt). Additionally, the level of JA-Ile after 6 hrs under salt stress increased significantly in comparing to its level after 1 hr under salt stress. On the other hand, no JA-Ile induction was observed in both JA-mutants under salinity stress in both 1 and 6 hrs. OPDA or oxo-phytodecatrenoic acid is a primary precursor of jasmonic acid with a regulatory signalling effect. The level of OPDA was found to be significantly higher in WT leaves (272.33 and 354 pmole/gFwt) after 1 hr and 6 hrs, respectively. JA-biosynthesis mutants did not show induction of OPDA in response to salt stress comparing to control conditions in both 1 hr and 6 hrs time points. Additionally, there

was no a significant difference between the level of induced OPDA in case of WT in both 1 hr and 6 hrs (Figure 22 C).

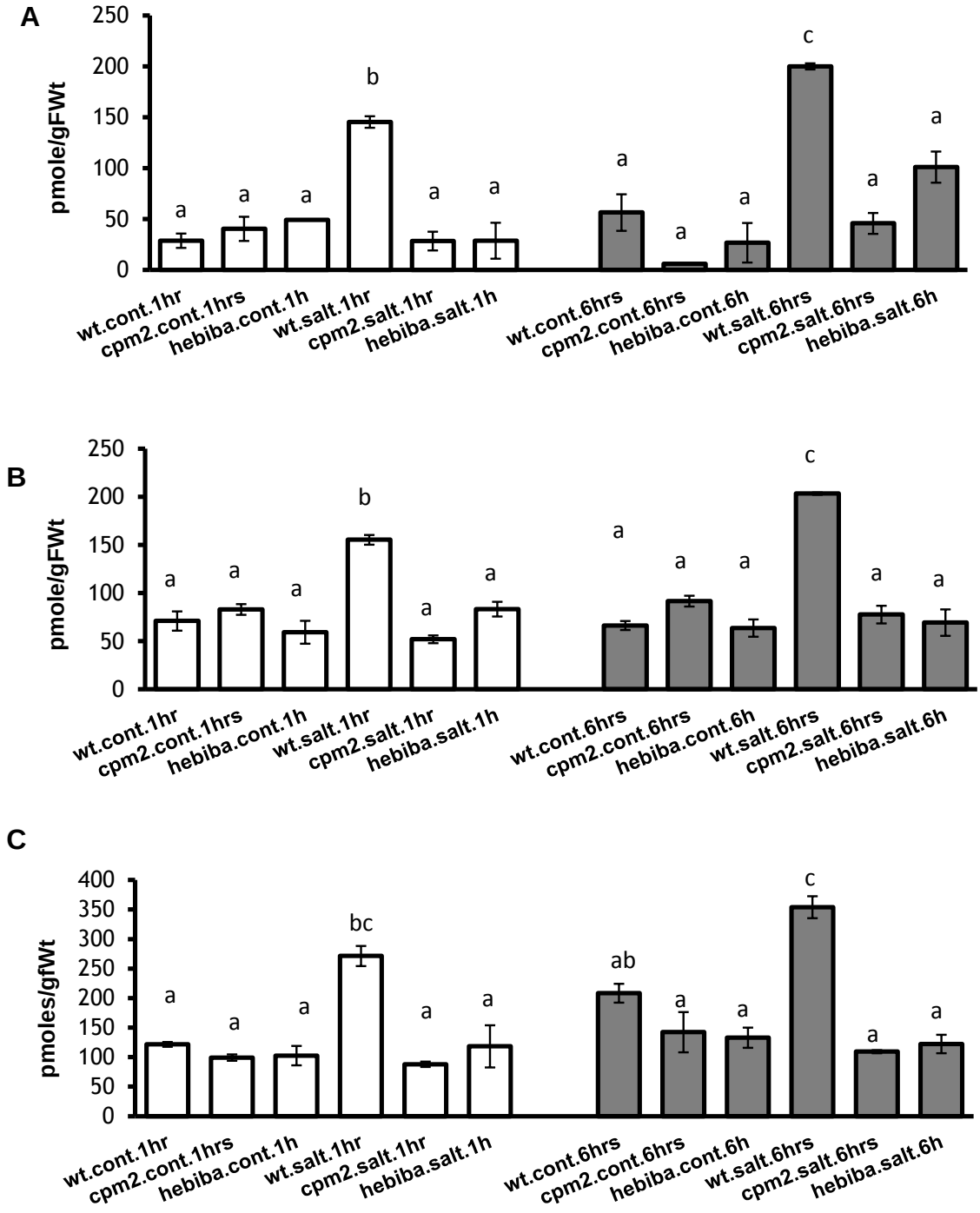

Figure 22. The level of Jasmonic acid (JA) and jasmonate isoleucine (JA-Ile) in two time points, 1 hr (blank bars) and 6 hrs (gray bars). A) Jasmonic acid level (JA). B) Jasmonte Isoleucine (JA-Ile). C) oxo-phytodecatrenoic acid (OPDA). Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

3.7.2 JA-mutants differ from WT in ABA production profile

The endogenous level of ABA was measured in leaves of JA-mutants and WT under salt stress after two time points, 1 hr and 6 hrs. After 1 hr, under control conditions there was no difference between WT and mutants ABA levels. However, the mutants showed a significant increase of ABA level in response to salinity comparing to its levels in control conditions. In case of cpm2, ABA concentration was 919.33 and 194.33 pmol/gFW, salt and control respectively. In hebiba, it was 647 and 235 pmole/gFW, salt and control respectively. While in case of WT, ABA level between control and salt stress was not significantly different. Nevertheless, we did not find significant differences between the WT, cpm2 and hebiba in terms of ABA level under salt stress.

After 6 hours, our data appeared a similar tendency to the findings after 1 hour. Nevertheless, it is found that the level of ABA was significantly higher in hebiba than WT under salt stress condition (1122.33 and 571 pmol/gFW, respectively), while ABA level in case of WT and cpm2 under salt stress was statistically not different. Generally, ABA levels was only found to be increased by time in case of hebiba, 647 and 1122.33 pmol/gFW for 1hr and 6 hrs, respectively.

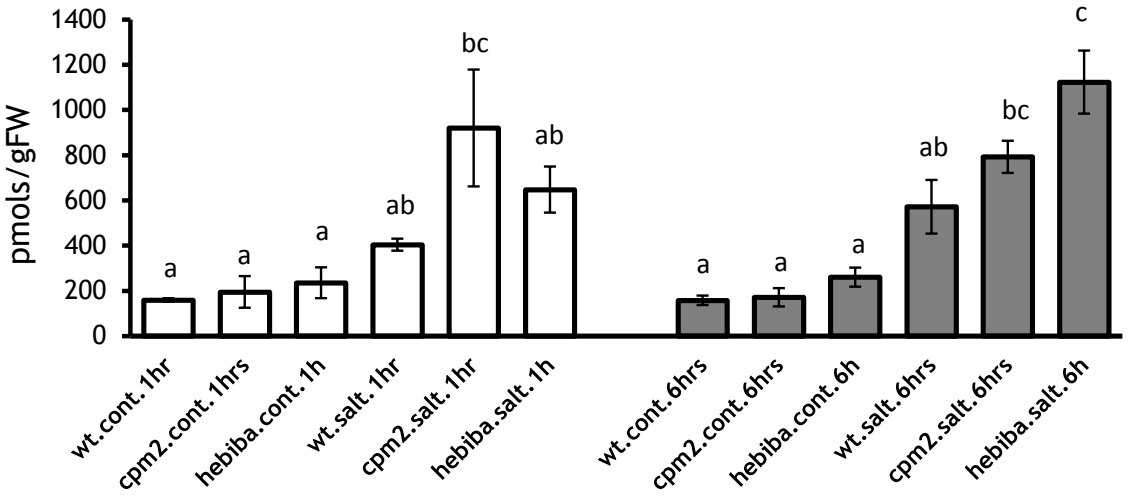

Figure 23. The endogenous level of Abscisic acid (ABA) in two time points, 1 hr (blank bars) and 6 hrs (gray bars). Values represent the mean of at least three independent experiments +\- SE. Means followed by different letters among treatments are significantly different, according to ANOVA single factor test with respect to Tukey's Honest Significant Difference (HSD) test ($P<0.05$).

3.7.3 Nitric oxide (NO) level might be contributed in the story

Since we have observed a higher level of OsNR gene expression in the mutants after 24 hrs of salt stress, we wanted to investigate the production of nitric oxide (NO) in the leaves of rice plants in order to investigate its incorporation - as a new member of plant growth regulators- in salt stress physiology. Consequently, we attempted to estimate qualitatively NO production in the guard cells using the fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2DA), which exhibit a high specificity to NO. Afterwards samples were examined by confocal fluorescent microscope (DAF-FM DA excitation 490mm, emission 525 nm) in LSM 710 NLO (Carl Zeiss, Germany).

As shown in Figure 24, the green fluorescence, indicating the presence of endogenous NO, was found in the guard cells of the examined leaf segments of both cpm2 and hebiba, nevertheless, an increased NO-specific fluorescence in the surrounding epidermal cells of both WT and JA-mutants under salt stress could be observed, although it is stronger in case of stressed mutants. Generally, we observed that the bundle-shape guard cells of cpm2 and hebiba showed relative stronger fluorescence comparing to the WT. Two powers of magnification were used (25X and 63X oil lenses) in order to focus on the intensity of the fluorescent dye in the guard cells that surrounding the stomata. As a control, the examination was performed in leaf segments of plants under control conditions (deionized water). The fluorescence in control conditions was very weak and not seen in all samples.

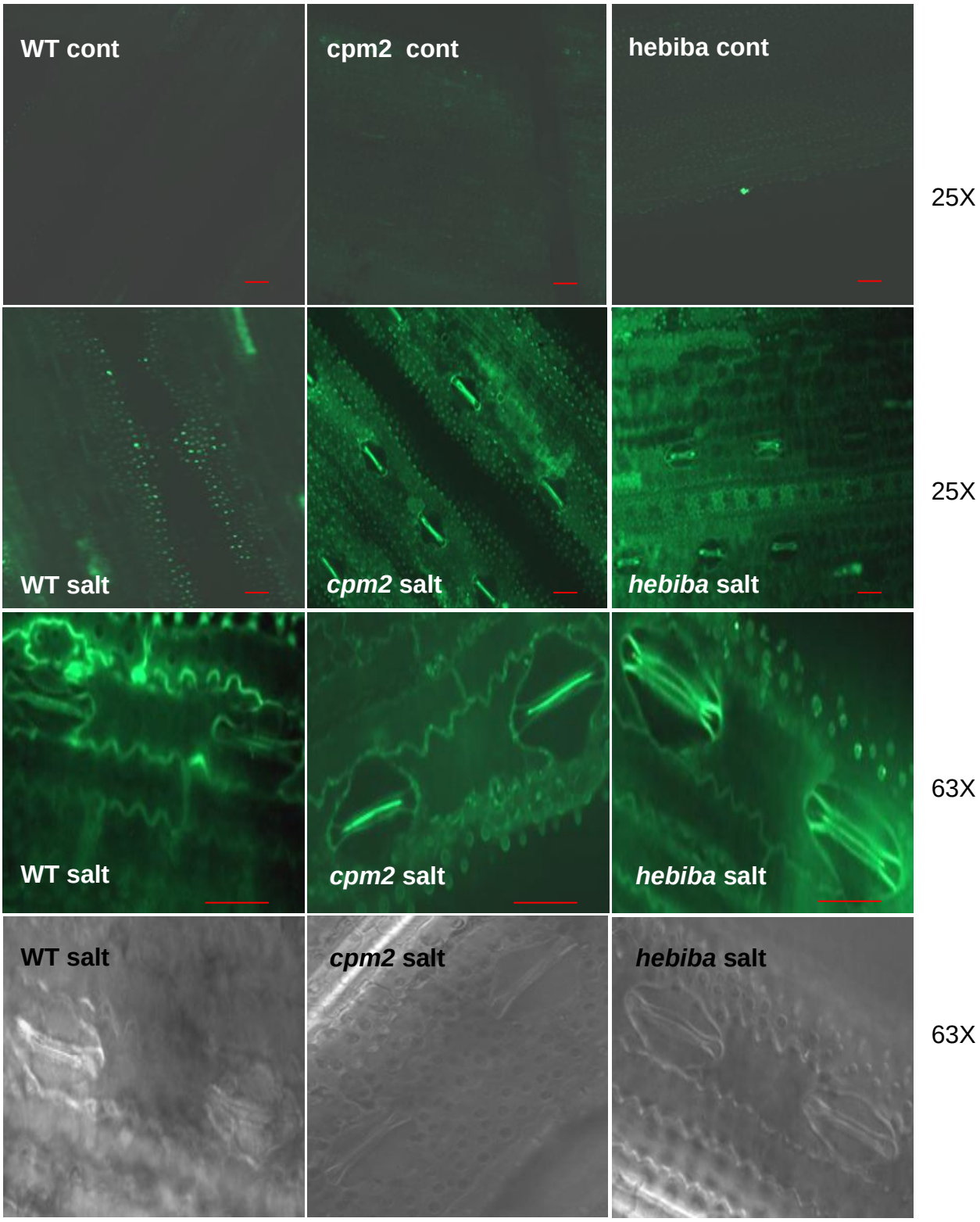

Figure 24. Confocal laser scanning microscopy of guard cells of third leaf mature part of rice plants under control and salinity stress conditions after 24 hrs. Leaves segments treated with 50µM DAF-2DA solution and washed with washing buffer. Two powers of magnification were used (25X and 63X oil lenses). Bar equal to 20µm.

3.8 Summary of results

The produced data in this study was intended for comparing the relative changes that involved in salt-stress adaptation mechanisms of two jasmonic acid deficient mutants lines (cpm2 and hebiba) to their wild type background rice cultivar Nihonmassari in the age of seedling stage. During this work, morphological, physiological, biochemical and molecular aspects were used as an attempt to make the output of this comparison considerable and of useful meaning.

The data obtained in this study showed the following observations:

1) The morphological investigation revealed that the damage symptoms that triggered by salt stress as leaf tip decolourization and whole leaf wilting were found to be less announced in the mutants plants comparing to WT. Additionally, the root length of JA- mutants was measured to be longer than wild when the seeds allowed to be germinated for 7 days in dark condition on phytoagar medium containing 62.5 and 125 mM of NaCl.

2) The level of accumulated Na+ ions was measure using atomic absorption spectrophotometer. Interestingly, the leaves of the mutants showed less uptaken sodium ions than WT. However, the sodium content in roots was similar statistically in both of WT and mutants (Figure 9). Furthermore, the damage effect of accumulated sodium ions on the chlorophyll and photosynthesis was investigated in terms of total chlorophyll content which was decreased in WT salt stressed leaves, while the mutants kept it in the range of control healthy plants .

3) The degree of oxidative damage was estimated through MDA levels (Malondialdehyde) which was lower in the mutants than WT under salinity stress. H_2O_2 micromolar concentration within plant shoot tissue was also measured and found to be less in JA-mutants leaves comparing to WT.

4) The produced antioxidants compounds in response to salt stress were also investigated by measuring the level of soluble proline, total phenolics content

(TPC) and total flavonoids content (TFC). JA-mutants produced less soluble proline than WT, although both of them show a strong increase in response to salt. On the other hand, TPC and TFC were statistically similar in mutants and WT, although their levels dramatically reduced in all salt stressed rice plants.

5) The activity of antioxidants machinery in rice plants were studied including its two branches, non-enzymatic and enzymatic ROS scavengers. For the non-enzymatic antioxidants, the crude extract of JA-mutants scavenge in vitro produced ROS better than WT as mutants recorded lower IC_{50}%, also for the antioxidants enzyme activity, many enzymes were more active in the mutants protein extract as SOD, POD, GR and GST, while APX was lower in the mutant and CAT showed similarity.

6) The expression profile of some gene candidates was studied and it is found that, OsNHX1 (Na^+ compartmenting in vacuoles), OsSAT (sulphur assimilation), OsOXO4 (H_2O_2 production form oxalic acid), OsJAZ13 (transcriptional regulation of JA-response genes) were found to be less expressed in JA-mutants leaves under salt stress comparing to WT, while the mutants expressed more both of OsNCED5 (ABA synthesis) and OsNR (Nitric oxide synthesis).

7) The profile of hormones production in response to salt stress was done. jasmonates (JA, JA-Ile and OPDA) were increased in WT leaves only but not in the mutants. For ABA, its level was similar in both of WT and JA-mutants after 1 hr, however, after 6 hrs ABA level in hebiba was higher than WT but not cpm2. Although, in both time points we observed that JA-mutants were able to accumulate more ABA in response to salt stress comparing to control conditions where ABA levels were statistically similar with all samples under salt stress. Unlike WT, JA-biosynthesis mutants showed more fluorescence of NO-binding specific dye (DAF-2DA) in case of many guard cell of the third leaf.

4- Discussion

Jasmonates are lipid-derived plant hormones regulating plant vegetative and reproductive growth, defense responses against abiotic stresses (UV light and ozone), insects and necrotrophic pathogens (Acharya and Assmann, 2009; Katsir *et al*., 2008a). However, the understanding of its role in mediating salt stress responses especially in rice- as a model plant for monocots - is still partially fragmented. Rice is the most important staple food crop on earth as half of mankind depends on it for their daily calorie intake, but unfortunately it is very sensitive to salinity stress at seedling stage, its height, root length, emergence of new roots, and dry matter decreases significantly under salinity (Pearson *et al* 1966, Akbar and Yabuno 1974). One of the challenges limiting investigations of jasmonate function in salinity stress physiology and biochemistry in rice is the unavailability of well-characterized biosynthetic rice mutants. Riemann *et al*. (2013) successfully isolated and characterized two Jasmonate-deficient mutants (cpm2 and hebiba) which were used in this study. The intension of the current work is to compare the JA biosyntheis mutants to their WT under salt stress on the level of morphology, physiology, biochemistry and molecular biology in order to shed more light on the role of jasmonic acid during adaptation to salt stress. Our findings revealed that jasmonates contribute to salt stress responses in rice.

4.1 Improved salt tolerance in rice jasmonate biosynthesis mutants

Unexpectedly, both JA-mutants showed a less salt-sensitive phenotype compared to their wild type background cultivar Nihonmasari. Phenotypic appearance of a plant can indicate the level of salt tolerance- According to our observations in this context, the mutants showed less salt damage symptoms with respect to second and third leaf wilting (Figure 7), additionally the root length of the mutants under middle and high NaCl concentrations were significantly longer than that of the wild type (Figure 8). Furthermore, the content of total chlorophyll of WT was significantly reduced in response to salt stress, while the mutants chlorophyll level was similar in stress and control conditions (Figure10). Our observation is supported by a report by Wilson (2007) who stated that treatment of rice plants with exogenous JA not only did not ameliorate the negative effect of salinity on the level of root dry weight and shoot dry weight but

also caused a strong reduction in those growth parameters. These results together indicate that the mutants suffer less than WT from the toxicity effect of high salt stress.

In order to investigate the reason(s) behind this observation, we analyzed the amount of accumulated sodium ions within roots and leaves. Interestingly, as shown in Figure 9, the mutants accumulated smaller amounts of sodium ions in leaves while roots were similar compared to the wild type. One of the most important parameters that distinguish the rice salt tolerant cultivars, as Pokkali, is its ability to uptake less sodium ions in leaves, while IR29, a well acknowledged salt sensitive rice cultivar, accumulated massively larger amount of sodium ions in the leaves when exposed to salt stress (Golldack *et al*, 2003 and Kader and Lindberg, 2005). Therefore, we can suggest that one of the main reasons which are behind the less salt-sensitive phenotype of JA biosynthesis mutants is its trait to take up lower amounts of sodium ions in the photosynthetic tissues using strategies that will be discussed at the end of this chapter.

In current reports the function of jasmonate in salt stress is under debate. On the one hand, exogenous application of JA leads to the reduction of sodium uptake in rice salt sensitive cultivars but not in salt tolerant cultivars (Kang *et al*, 2005). On the other hand, it was also reported that exogenous application of JA leads to a massive accumulation of sodium ions in rice plants (IR29, salt sensitive rice cultivar) resulting in sodium concentration in the milimolar range in case of salt stress treatments (Wilson, 2007). For maize Shahzad (2011) reported that exogenous application of JA leads to an increased movement of sodium ions from root to shoot compared to non-JA-treated plants. However, JA treatments reduced the amount of sodium ions in both roots and shoots.

4.2 Reduced oxidative damage in JA mutants in response to salt stress

Salt stress can generate ionic stress and osmotic stress in plant cells leading to the production of toxic levels of reactive oxygen species (ROS) (hydrogen peroxide-H_2O_2, superoxide radical-$O_2^{\cdot-}$, hydroxyl radical-$HO^{\cdot}$), which cause an effect referred to as oxidative stress. Oxidative stress leads to the damage of membranes lipids, proteins and nucleic acid (Pang and Wang, 2008). Initially ROS were thought to be toxic

byproducts of aerobic metabolism, but recently have been acknowledged to be central players in the complex signaling network of cells (Mittler *et al*, 2011). They can be generated enzymatically, e.g by the membrane located NADPH oxidase, as signaling molecules or as toxic compounds at several locations in the cell, e.g. in the chloroplast when photosynthesis is deregulated.

We applied in this study a set of experiments leading to observations supporting the hypothesis that JA biosynthesis mutants suffer less from the oxidative stress which is initiated from salinity stress. MDA is an important intermediate in ROS scavenging and considered to be an indicator of the extent of oxidation damage under stress (Apel and Hirt, 2004 and Borsani *et al*, 2001). MDA level was estimated under salinity, and we found MDA levels are significantly lower in the leaves of mutants compared to the wild type (Figure 11 A). A similar result was reported by Vaidyanathan *et al* (2003) where MDA level was lower in Pokkali (salt tolerant rice cultivar) compared to Pusa Basmati (a salt sensitive rice cultivar) which showed a higher level under salt stress. Additionally, the level of hydrogen peroxide (H_2O_2) was measured (Figure 11B). Hydrogen peroxide is potentially reactive oxygen, but not a free radical (Halliwell *et al*, 2000). Compared to the other ROSs, H_2O_2 is relatively innocuous, however, the risk results from its non-enzymatic conversion to hydroxyl radical anions ($HO^{\cdot}$) which reacts strongly and rapidly (in less than 1 µs) with proteins, lipids and DNA, causing cell membrane damage (Ishada *et al*, 1999). H_2O_2 accumulated to toxic oxidative levels under salinity stress, also its level increased by raising the concentration of NaCl in rice and soybean (Mandhania *et al*, 2006, and Weisany *et al*, 2012).

In order to gain some knowledge about the level of non-enzymatic antioxidants which help in removing ROS under stress conditions and lead to mitigating levels of MDA and H_2O_2 in cpm2 and hebiba leaves, we have estimated the amount of some non-enzymatic antioxidants such as soluble proline, total phenolics and total flavonoids (Figure 12 A, B and C). Soluble proline is considered as an osmoprotectant, probably associated with osmotic regulation and membrane stability under stress (Maggio *et al*, 2000). However, its role under salt stress is not an absolute fact, as there are many reports, e.g. Lutts *et al* (1996) and Poustini *et al* (2007), which showed that proline may

not be involved in osmotic adjustment under salt stress in rice and wheat. On the other hand its accumulation is consistent in rice under salinity stress (Garcia *et al*, 1997 and Hoai *et al*, 2003). The level of soluble proline was found to be dramatically increased in both wild type and mutants under the effect of salt stress, nevertheless, its amount was significantly lower in the mutants, and that could be correlated to the lower amount of accumulated sodium ions in the mutant leaves. Therefore its accumulation has an injury effect rather than being involved in stress tolerance as proposed by different groups (Schobar *et al*, 2010, Poustini *et al*, 2007).

Phenolics are diverse secondary metabolites (flavonoids, tannins, esters and liginin) which are abundantly found in all plant tissues. They are processing antioxidants properties because of their strong capacity to donate electrons or hydrogen atoms (Grace and Logan, 2000, and Sharma *et al*, 2012). In the current study we estimated total phenolics content (TPC) and total flavonoids content (TFC) in both control and salt stress conditions. Both of TPC and TFC were found to be significantly diminished in response to salt stress in case of wild type and JA-mutants, additionally, neither cpm2 nor hebiba showed significant differences of TPC and TFC amounts compared to wild type under salt stress.

According to our observations, we may speculate that in case of rice, as a very salt sensitive glycophyte, salinity stress-induced tissue damage leads to breakdown of TPC and TFC as main components in plant cell walls. It seems that TPC and TFC are not a main strategy in conferring oxidative stress in rice seedling. There are some reports stressing that the accumulation of total phenolics was observed to reach twice as high in halophytes species such as Tamarix gallic comparing to glycophytes (Nigella sativa) (Ksouri *et al*, 2012 b). Furthermore, under exposure to salt stress conditions, the leaves of salt sensitive glycophyte species such as Lactuca sativa showed a reduction in its polyphenolics contents, while the salt tolerant species as Solanum pennellii LA716 accumulated more polyphenolics in its leaves (Waśkiewicz *et al*, 2013).

Plants are equipped with an array of enzymatic and non-enzymatic antioxidantive molecules to alleviate cellular damage caused by ROS (Foyer and Noctor, 2000). In order to study the capacity of plant extracto to scavenge ROSs non-enzymatically we

tested extensively crude leaf extracts of the mutants and wild type. DPPH is a stable nitrogen-centered free radical, and its color changes from violet to yellow when reduced by the process of hydrogen or electron donation (Liu *et al*, 2008). The higher the DPPH scavenging activity the better the antioxidative ability of the tested extract. The salt stressed mutants, as shown in Figure 13, showed a better inhibition activity and lower IC% compared to the wild type, hence the antioxidative activity of JA biosynthesis mutants was better than that of the wild type. In Arabidopsis thaliana, as reported by Nakabayashi *et al* (2013), the antioxidantive activity of the crude extract of anthocyanin overexpressing lines exhibited greater radical scavenging activity to be two folds higher than those from wild type under drought and oxidative stresses in DPPH assays.

We investigated the non-enzymatic ability of wild type Nihonmasari and the JA-mutants (cpm2 and hebiba) crude extract to detoxify some ROS molecules as superoxide anion, hydrogen peroxide and hydroxyl radical under salt stress time course. In case of antioxidative power against superoxide anions toxicity, we showed that the crude extract of all samples under control and salt stress conditions were able to scavenge the in vitro produced superoxide anions in a dose dependent manner. Nevertheless, its antioxidantive activity against $O_2^{\cdot -}$ was much weaker under salinity stress relative to control conditions, and this fits to our observation that the content of total flavonoids and total phenolics compounds were found to be dramatically diminished in salt stress compared to control. Furthermore, the crude extract of both JA biosynthesis mutants were able to detoxify $O_2^{\cdot -}$ much better than that of the wild type under salt stress as shown in Figure 14D suggesting a stronger antioxidative capacity under salt stress compared to wild type leading to lower level of toxic $O_2^{\cdot -}$ molecules. Recently, Chawala *et al* (2013) reported that Pokkali (salt-tolerant rice cultivars) in comparison to the salt-sensitive rice cultivars IR-28 accumulated clearly lower amounts of superoxide radicals in response to salt stress triggered by 100mM NaCl.

Multiple antioxidantive enzymes were investigated in this work. The activity of Superoxide dismutase (SOD) diminished in WT leaves after salt stress, while it was stable before and after salt stress in both of JA biosynthesis mutants (Figure 17). Superoxide dismutase is an enzyme that catalyzes the dismutation of superoxide

radicals ($O_2^{\cdot -}$) to hydrogen peroxide and water (McCord and Fridovich, 1969).Therefore, it is suggested that the enzymatic scavenging activity of superoxide is enhanced in JA biosynthesis mutants. Catalase (CAT) is one of the important enzymes that can scavenge large amounts of hydrogen peroxide; it can decompose H_2O_2 directly to give water and oxygen (Aebi, 1984). As illustrated in Figure 18 A, CAT activity was found to be similar under salt stress in JA biosynthesis mutants and wild type. Nevertheless, we observed in general a strong reduction in CAT activity in rice shoots on its exposure to salinity stress in all treatments. This observation was reported in rice in a majority of reports, like Shim *et al* (2003) and Sahu *et al* (2010) without a clear explanation. Ascorbate peroxidase (APX) catalyses the reduction of H_2O_2 to water and oxygen using ascorbate as electron donor (Asada, 1999). APX activity was increased in response to salinity in the shoots of wild type but not the JA biosynthesis mutants (Figure 18 B). In salt stressed plants, the APX activity was dramatically decreased JA biosynthesis mutants compared to the wild type. This was probably due to the lower amount of sodium ions that accumulated in leaves of the mutants, as Lee *et al* (2013) reported a higher level of APX activity in rice leaves of IR29 (massive sodium ions accumulative rice cultivar), while in case of Pokkali (accumulates less sodium ions), APX activity was significantly diminished.

Peroxidase includes in its widest sense a group of specific enzymes as NAD-Peroxidase, NADP-peroxidase, fatty acid peroxidase and others, and a group of very non-specific enzymes from different sources which are simply known as POD. POD catalyses the dehydrogenation of some antioxidantssuch as phenolics, aromatic amines and others in order to breakdown and/or produce hydrogen peroxide and ROS (Malik, and Singh, 1980, and Donald, and Cipollini, 1997). The POD activity was induced by salinity in the mutants only, hence the mutants showed a higher activity level than wild type, and this might lead to a better scavenging activity for hydrogen peroxide which could be the reason behind the lower H_2O_2 level in the salt stressed JA biosynthesis mutants (Figure 19). The involvement of glutathione metabolism in mediating salt stress in the mutants was also provided in this study through measuring the activity of GR and GST (Figure 20 A and B). Both of the glutathione related antioxidative enzymes showed a higher activity in the mutants (in case of GR, this holds true only for hebiba).

Glutathion reductase and Glutathion-S-transferase were proposed to confer protection under various stress conditions, biotic and abiotic, by detoxifying endogenous pant toxins that accumulate as consequences of increased oxidative stress (Marrs, 1996, and Yousuf *et al*, 2012) which might be the case in the cpm2 and hebiba.

4.3 Evidence for jasmonates as regulators of NHX1 and ABA modulators

Thoroughly, we tried to achieve a solid explanation for the physiological, biochemical and molecular changes triggered in the jasmonate biosynthesis mutants which lead to the observation of better adaptive response under salt stress. We investigated the expression profile of several genes sharing in different aspects in molecular trafficking under salt stress conditions in two different time points (24 h and 72 h) in leaves. One mechanism involves removal of sodium ions from cytoplasm by its transport into vacuoles. This transport is catalyzed by Na^+/H^+ exchanger tonoplast antiporter protein which is coded by NHX1 gene (Qiu *et al*, 2004). OsNHX1 gene expression was found to be elevated after exposure to salt stress in both wild type and JA biosynthesis mutants. However, in both time points, the abundance of its transcripts was lower in the mutants that accumulated less sodium ions compared to the wild type (Figure 21). Therefore, the sequestration of sodium ions from cytoplasm into vacuoles through NHX1 exchangers might be not a main strategy for better salt stress adaptations in case of JA biosynthesis mutants, while the lower expression of NHX1 gene in the mutants could be due to the lower concentration of Na^+ ions in the cytoplasm of leaf cells. This conclusion is in agreement with the observation of Li *et al*, 2008 and Khedr *et al*, 2011 who described that NHX1 expression seems to be increased by raising the concentration of NaCl in both rice and Atriplex. Nevertheless, this is contradictory to the reports indicated a lower sodium contents in transgenic rice plants overexpressing OsNHX1 (Liu *et al*, 2010).

As we have done our measurements in NaCl solution without any other nutrient additions, so the seedlings are under K^+ starved conditions in both control and salinity treatments. Under such conditions, many nonhalophytic plants - as rice - can utilize Na^+ for K^+ non-specific metabolic and physiological functions as stomatal functions probably because sodium in such case is highly mobile and energetically cheap, although it is

toxic at high concentrations (Hageman, 1984, Newbould, 1989 and MacRobbie, 2006). As JA biosynthesis mutants showed lower levels of OsNHX1 gene expression, it is proposed that the activity of such vacuolar antiporters is smaller than in the wild type, leading to less turgid / sodium containing vacuoles which could cause impaired stomatal opening compared to wild type. this conclusion is in line with a recent study by Andrés *et al* (2014) who observed that nhx1 Arabidopsis mutant plants survived longer under water deprivation because it transpired less per leaf area unit during the day, thus consuming less soil water.

In order to investigate the effect of salt stress on signaling of jasmonates (JA, MeJA and JA-Ile), the relative expression of JAZ13 gene was quantitatively determined. JAZ or Jasmonate ZIM-domain proteins are transcriptional repressors and play a critical role in mediating jasmonate signaling under developmental and stress conditions (Staswick, 2008 and Acharya and Assmann, 2009). Expectedly but interestingly, JAZ13 gene expression was strongly upregulated in response to salt stress in both time points, in particular after 72 hrs in the wild type while JA-mutants responded much weaker in this context. This observation is going along with Ye *et al* (2009) and Zhu *et al* (2012) who reported that some members of the JAZ family are involved in salt stress response in rice and its overexpression in soybean or rice increased salt and alkali stress tolerance. Furthermore the endogenous level of jasmonic acid, and its bioactive members as jasmonate and its precursor OPDA in both WT and JA biosynthesis mutants were analyzed in shoots under control and salt stress conditions. Our results indicated that levels of jasmonic acid, JA-Ile and the precursor OPDA were found to be elevated in response to salinity in the wild type, while it was not changed –expectedly- in the mutants (Figure 22). Intriguingly, OPDA levels were elevated more clearly after 6 h in salt stress as compared to JA and JA-Ile. As it is known that OPDA by itself has hormonal activity (Stintzi *et al*. 2001, Taki *et al*, 2005) this might hint to a function for OPDA in salt stress. Very recently a similar observation was made for drought stress in Arabidopsis, were OPDA but not JA was found to be elevated (Savchenko *et al*. 2014). The level of jasmonic acid was found to be increased in response to salt stress in tomato (Pedranzani *et al*, 2003) or in rice (Moons *et al*, 1997), while other reports mentioned that jasmonic acid level was not changed in both salt-tolerant and salt-

sensitive maize shoot cultivars (Shahzad, 2011). These results may support the hypothesis that the role of jasmonic acid in salt stress is mainly related to the genotype and its overall degree of sensitivity to salinity, so we state that screening many halophytes and glycophytes plants with respect to jasmonates accumulation in both shoots and roots will enhance our knowledge about its role in salt stress adaptation.

As one of most salt-sensitive glycophytes, rice has very limited effective strategies for dealing with salt and do not grow well on saline soil (Galvan-Ampudia and Testerink, 2011). Therefore, lowering the amount of uploaded sodium in the transpiration stream is a vital approach for more adaptation under salt stress in rice seedlings. As previously shown in Figure 9, the sodium contents in roots was similar, and was different only in shoots, hence we can conclude that the root-to-shoot sodium transfer was less in JA biosynthesis mutants than WT. In order to investigate the hypothesis that this changing root-to-shoot transfer was due to the effect of stomatal closure on the transpiration stream, we have checked two marker genes of pathways regulating stomata movement, namely abscisic acid (ABA) and nitric oxide (NO) (Figure 22). The role of ABA was investigated through measuring the expression of one of the ABA key biosynthesis enzymes OsNCED5 (9-cis-epoxycarotenoid dioxygenase). Interestingly, we found its expression was higher in the mutants especially after three days of salt stress. Additionally, the endogenous ABA level was analyzed in leaves of wild type and JA biosynthesis mutants. As shown in Figure 23, after 1 hr of salt stress, the level of ABA was similar between WT and JA-mutants. While after 6 hrs, ABA level was higher in hebiba compared to WT, but not cpm2. Although the level of ABA in both WT and JA-mutants seems to be comparable, we observed in both time points (1 h and 6 h) salinity stress enhanced ABA levels in JA biosynthesis mutants compared to the wild type which might indicate that biosynthesis of ABA is induced faster in the mutants. This would be in line with the antagonistic relationship between JA and ABA in rice roots as reported by Moons *et al*, 1997. Additionally and equally important, the response to ABA is a vital step in stomatal closure and inhibiting opening of stomata. NO is one of the hormones which was recently reported to be downstream of ABA and functional in the regulation of stomatal closure. Nitrate Reductase (NR) was noticed to be one of the

most candidates that could be related to the generation of nitric oxide in plants (Desikan *et al*, 2002).

JA biosynthesis mutants accumulated a higher level of the transcripts of Os02g0770800 gene, referred it as Os semiNR, compared to the wild type after one day of salt stress. The produced NO amount in response to salt stress was measured histochemically in stomatal guard cells (Figure 24), we observed that the guard cells of JA biosynthesis mutants produce more NO than WT. Together; we can suggest that JA-mutants accumulated less sodium ions within the shoots due to the faster guard cells movements that lead to a faster stomatal closure. ABA is a plant hormone and one of the foremost signaling molecules in plants, which plays versatile functions in regulating many developmental processes and adaptive stress processes (Culter *et al*, 2010). A lot of extensive studies supporting its role of ABA in the mechanisms of stomata closure under normal and stress conditions. Nitric oxide (NO) was introduced as a co-mediators for ABA in stomatal closure, also recently it was reported that treating wheat plants with SNP (sodium nitroproside) as NO donor, results in the enhancement of adaptive response to drought stress through the induction of stomatal closure (Neil *et al*, 2002, and García-Mata and Lamattina, 2014). In summary, based on our results we suggest that jasmonates may affect the biosynthesis of NO and in this way alters stomata closure.

Taken together, and in order to link the ideas previously discussed, a graphical model was suggested and shown in Figure 25. The model indicates the relationship between the less sensitive salt stress phenotype in JA biosynthesis mutants and relative physiological and molecular changes in response to salt stress. When exposed to salt stress, roots of both WT and mutants seedlings accumulated similar amount of Na^+ ions as shown in Figure 9. Because the mutants accumulated less sodium ions in leaves compared to the wild type, the transfer of sodium ions from roots to shoot was reduced in case of JA biosynthesis mutants. This conclusion could be tested in future through investigating the parameters controlling the speed of transpiration stream as opening and closing of stomata. Opening of stomata is controlled by guard cell vacuole size. By our finding that NHX1 transcripts are less responsive to salt stress in JA biosynthesis

mutants we have indirect evidence that also NHX1 activity in is reduced relative to WT after salt stress. Therefore, the vacuoles in JA biosynthesis mutants guard cell should be less turgid and partially closed in the first instance even before exposure to salt stress. First evidence for this assumption derives from a recent master thesis (Christina Manz 2014), in which it was found that the stomatal conductivity is decreased in the mutants compared to the wild type under normal growth conditions. Additionally and equally important, the expression profile of two important genes which are related to stomata aperture regulation, OsNCED5 (ABA synthesis key enzyme encoding gene) and OsNR (Nitric oxide rate limiting enzyme encoding gene) were investigated. Interestingly, both of them were found to be up regulated in the JA biosynthesis mutants (Figure 22). Although there is no strong evidence that the biosynthesis of ABA is significantly altered in the mutants the sensitivity to ABA might be affected, supported by a increased relative expression change of OsNCED5 in JA This point will be addressed in future e.g. by measuring expression levels of ABA-responsive genes in wild type and mutant plants. Nitric oxide is reported to be downstream to ABA and its role in stomatal aperture movement is now known.

NO was histochemically visualized and found to be to be more abundant in guard cells of JA biosynthesis mutants. Together, we can speculate that stomata of JA biosynthesis mutants have the tendency to close after a shorter period of time compared to the wild type when salt stress was triggered. This might be because of an increased sensitivity to ABA in the mutants because it is known that ABA signaling activates NO biosynthesis. Due to altered stomatal opening, the transpiration stream might be slower in the mutants leading to less root-to-shoot sodium ions transfer. Rice leaves, as photosynthetic-salt sensitive tissues, produce reactive oxygen species (ROS) under normal conditions, however, ROS are accumulated to toxic levels in response to salt stress as shown in Figure 4 (introduction chapter). As a result of transferring less sodium from roots to its leaves, JA biosynthesis mutants suffer less from oxidative stress as its ability to scavenge ROS was not totally damaged by Na^+ ions. On the other hand, the antioxidant scavenging power of the wild type was (enzymatically and non-enzymatically) relatively disarmed probably due to severe sodium toxicity.

Consequently, the wild type was observed to suffer from more severe salt stress damage symptoms as leaf wilting and leaf tip discoloration.

4.4 Conclusions

1) Improved salt tolerance in rice jasmonate biosynthesis mutants.
2) Reduced oxidative damage in JA mutants in response to salt stress.
3) The movement of stomatal aperture in jasmonates biosynthesis mutants seems to be impaired in terms of stomatal opening.
4) Jasmonates might be regulators of NHX1 and ABA modulators.

4.5 Outlooks

1) Production of transgenic rice plants overexpressing OsNR, OsOXO4 and OsSAT.
2) Investigating the response of those transgenic rice plants to abiotic stresses (drought, salt and alkalitiy) with respect to jasmonic acid.

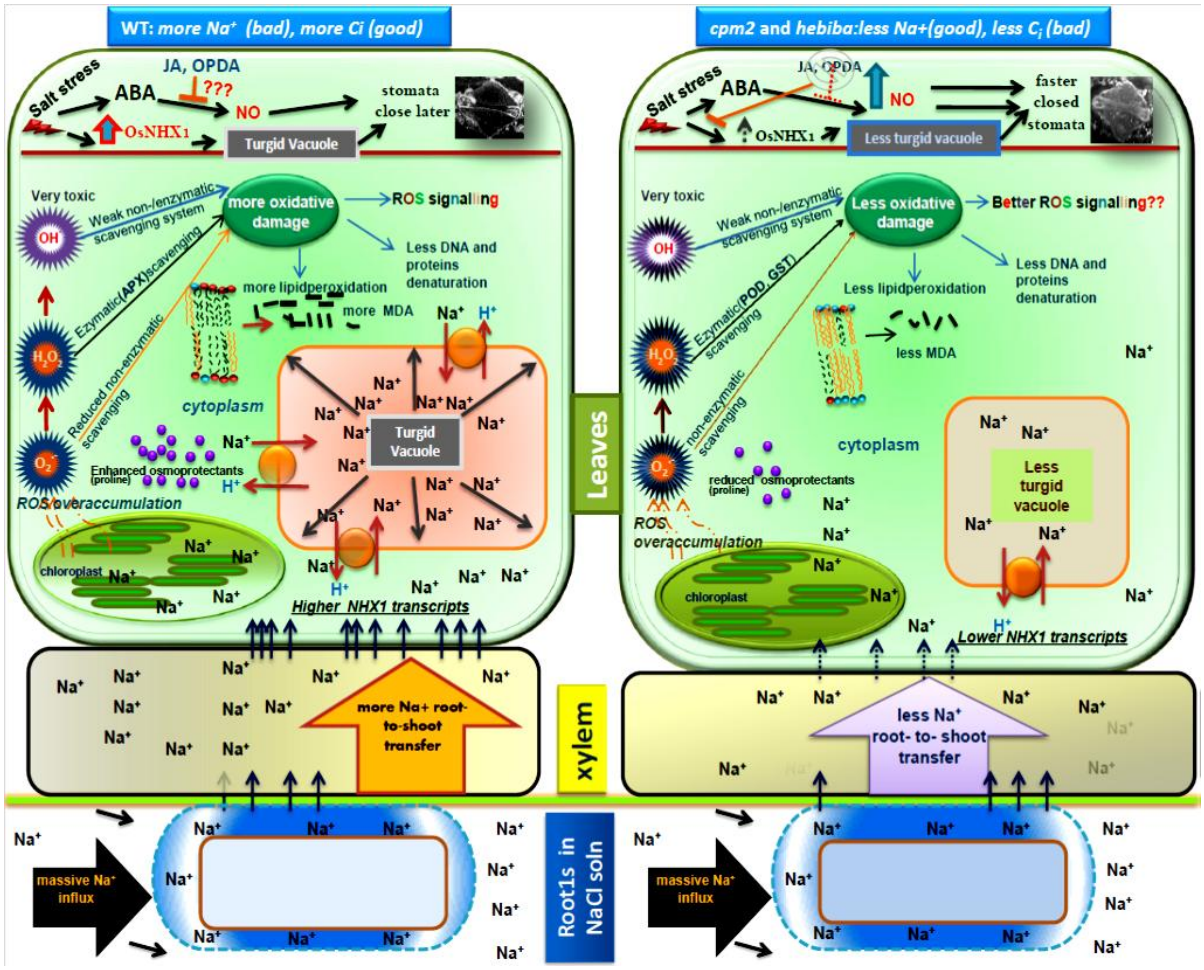

Figure 25. Graphical model representing physiological and molecular changes of two jasmonate-deficient biosynthesis rice mutants (*cpm2* and *hebiba*) compared to their WT in response to salinity stress (100mM NaCl for 24 and 72 hrs). The stomatal aperture in JA-mutants might suffer from impaired dynamic regulation in terms of stomatal opening leading to less sodium ions carried in the transpiration stream, and less internal CO_2 (C_i). The reasons could be 1) Active abscisic acid (ABA) signalling reflected by more production of nitric oxide (NO). 2) Lower activity of vacuolar H^+/Na^+ antiporters (NHX1) resulted in less turgid vacuoles under potassium starving conditions. Lower sodium content in JA-mutants caused less damage, and the antioxidative machinery was able to detoxify the produced reactive oxygen species (ROS) as superoxide ions ($O2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$), while in the wild type a excess ROSs could not be scavenged effectively.

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