Inactivation of Jasmonate signaling -

A route to salt stress adaptation in rice

Zur Erlangung des akademischen Grades eines

DOKTORS DER NATURWISSENSCHAFTEN

(Dr. rer. nat.)

der KIT-Fakultät für Chemie und Biowissenschaften

des Karlsruher Instituts für Technologie (KIT)

genehmigte

DISSERTATION

von

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Tag der mündlichen Prüfung:12.12. 2017

Die vorliegende Dissertation wurde am Botanischen Institut des Karlsruher Instituts für Technologie (KIT), Botanisches Institut, Lehrstuhl 1 für Molekulare Zellbiologe, von April 2014 bis December 2017 angefertigt. Teile der experimentelle Arbeiten, die in dieser Arbeit beschrieben sind, am International Rice Research Institute, Philippinen, durchgeführt.

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Karlsruhe, im November 2017

Preshobha K Peethambaran

ACKNOWLEDGEMENTS

I would like to take this auspicious occation to thank deeply all the people without whom my PhD journey wouldn't be possible.

First and foremost, I would like to express my sincere gratitude to Prof. Dr. Peter Nick for accepting me as his doctoral student for the past three and half years. His inspiring personality, enthusiasm, patience, motivation, as well as for his continuous support during each step of my research helped me to accomplish my goals.

Secondly, I am extreamly grateful to Dr. Michael Riemann for his advice and help during each and every steps and problems through my whole PhD and stay in Germany. His observations and comments helped me to establish the overall direction of the research and to move forward with investigation in depth. I sincerely appreciate countless suggestions and for critical reading of my thesis and manuscript.

I am thankful to Prof Klaus Harter, Dr. Uener Kolukisaoglu and Sabine Hummel from University of Tuebingen who conducted DPI-ELISA assay and provided me an opportunity to observe the technique there.

I thank Dr. Ajay Kohli and Dr. R.K Singh for giving me opportunity and space to work in their lab in International Rice Research Institute (IRRI), Philippines. This improved my knowledge in salt stress in rice, where I could do some experiments in the real field conditions. I also thank Karlsruhe House of Young Scientist (KHYS) for supporting this trip financially.

I appreciate the excellent work of our lab technicians Nadja Wunch, Sabine Purper and Ernst Heene as well as our Azubi Lena Draheim, Jonas Muller and Natascha for helping me through the molecular biology experiments.

My thanks to all my colleagues at Botanical Institute 1 for such a friendly and helpful working environment. I thank especially Dr. Manish Raorane, Dr. Vaidurya Sahi and Dr. Rohit Dhakaray for their constant support and advices during the period of my PhD study. I am also thankful to Dr. Qiong, Dr.Jan, Dr. Viktoria, Dr. Rita, Dr. Sarhar , Dr. Linda, Fatemeh, Dr.Lixin,

Dr. Xiaolu, Dr. Xiang, Hao, Ruipu, and Pallavi from whom I always learned many things and got unselfish help. My special thanks also to master/bachelor students for their coorporation.

I am grateful to the financial support of the Indian Council of Agricultural Research for funding this study. To Prof. Suresh.S. Honnappagol, Assistant Director General (EQR), ICAR and especially to Mr. Bhagwat Singh, Se. Technical Officer ICAR and other officials who immensely helped and managed to give the scholarship on time.

Last, but not the least, I want to express my heartiest gratitude and acknowledgement to my respected parents, my sister and brother for their unconditional love and support. I have to specially thank my husband Adarsh Ninan for his constant motivation, encouragement and being so understanding and being with me throughout this phase of my life. My family has been source of inspiration, dedication, discipline and determination without which this thesis could never been completed.

Preshobha K Peethambaran November 2017

CONTENTS

ABBREVIATIONS

BY-2: Tobacco Nicotiana tabacum L. cv. bright yellow 2

ABA: Abscisic acid

GFP: green fluorescent protein

AOS: Gllene oxide synthase

JA: Jasmonic acid

JA-Ile: (+)-7-iso-jasmonoyl-L-isoleucine

LOX: Lipoxygenase

OPDA: 12-Oxo-Phytodienoic Acid

JAR1: Jasmonate Resistant 1

COI1: Coronatine Insensitive 1

ROS: Reactive oxygen species

IAA: Indole-acetic acid

MeJA: Methyl jasmoante

VOCs: Volatile organic compounds

WT: Wild type

12OH-JA-Ile: 12-Hydroxy-jasmonoyl-isoleucine

12COOH-JA-Ile: 12-Decarboxy-jasmonoyl-isoleucine

SCF: SKP1, Cullin, F-Box-Protein

C2H2: Cystein2/Histidin2

JAZ: Jasmonate ZIM domain

STZ: Salt tolerance zinc finger

ZFP: Zincfinger protein

ZAT: Zinc finger protein of Arabidopsis thaliana

ZOS: Zinc finger of Oryza Sativa

ZUSAMMENFASSUNG

Reis (*Oryza sativa* L.) ist für mehr als die Hälfte der Weltbevölkerung das Grundnahrungsmittel. Dabei ist die Bodenversalzung einer der wichtigsten abiotischen Stressfaktoren, der die Produktivität und die Verfügbarkeit von Agrarflächen ernsthaft beeinträchtigt und somit einen großen sozioökonomischen Einfluss darstellt. Die Jasmonat-Pflanzenhormone, unter welchen man Jasmonsäure und seine Derivate zusammenfasst, wirken als Signalmoleküle, die eine entscheidende Funktion in biotischer und abiotischer Stresstoleranz und für die pflanzliche Entwicklung haben. In dieser Arbeit versuchten wir mit einer innovativen Strategie zu zeigen, dass Unterdrückung von Jasmonsäuresignalleitung zu einer Änderung von Jasmonatsensitivität und Antwort auf Salzstress führen kann, ohne negative Effekte von Jasmonatunempfindlichkeit wie verminderte Fertilität oder erhöhte Empfindlichkeit für Pathogene in Kauf nehmen zu müssen.

Um diese Strategie zu überprüfen, haben wir transgene Reispflanzen sowie transgene BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow-2) Suspensionzellen hergestellt, die das vollständige bzw. das am C-Terminus verkürzte (ohne die Jas-Domäne) OsJAZ8 Protein unter Kontrolle der salzinduzierbaren ZOS3-11 oder ZOS3-12-Promotoren exprimieren. Für die transgenen Tabakzellen konnten wir die Aktivität der Promotoren untersuchen und zeigen, dass die Induktion des *OsJAZ8*-Gens zu Jasmonat-insensitiven Phänotypen führte. Die Zellen zeigten eine verminderte Zellmortalität, verstärktes Längenwachstum und eine Abnahme der Zellzyklusdauer und dadurch eine bessere Leistung insbesondere unter MeJA Behandlung, aber auch unter Salzstress. Interessanterweise hat die Unterdrückung von Jasmonsäuresignalleitung auch zu einem Anstieg der Responsivität gegenüber Auxin in transgenen BY-2 Zelllinien im Vergleich zum Wildtyp geführt, was zeigt, dass Jasmonat die Auxin Responsivität modulieren kann.

Transgene Reiskeimlinge zeigten eine bessere Toleranz gegen Salzstress, welches mit einem erhöhten Gehalt von *OsJAZ8* Transkripten in ZOS3-11::JAZ8-Linien und OsJAZ8ΔC Transkripten in ZOS3-11::JAZ8ΔC-Linien im Vergleich zum Wildtypen korrelierte. Die

transgenen Reislinien zeigten keinerlei phänotypischen Unterschiede unter Kontrollbedingungen. Der Gehalt an Transkripten von *OsJAZ8* und *OsJAZ8ΔC* war jedoch erhöht.

Im zweiten Teil dieser Arbeit wurden die Proteine ZOS3-11 und ZOS3-12 näher charakterisiert. Es handelte sich um C2H2 Zinkfinger-Transkriptionsfaktoren mit Homologie zu STZ von *Arabidopsis* und die Expression ihrer Transkripte war durch Salz in Abhängigkeit von Jasmonat induzierbar. Transiente Expressionsanalysen mit GFP-Fusionsproteinen zeigten, dass die Transkriptionsfaktoren vorzugsweise im Zellkern lokalisiert sind, obschon ihnen eine Kernlokalisationssequenz fehlt. Beide Proteine binden eine A(C/G)T-Sequenz und besitzen eine Repressorfunktion.

Eine frühere Studie, in der für die Jasmonat-Biosynthesemutanten (*cpm2* und *hebiba*) eine verbesserte Salztoleranz im Vergleich zum Wildtyp beschrieben wurde, wurde erweitert durch eine ausführliche Zeitverlaufsstudie, in der die Aufnahme von Natrium- und Kaliumionen im Vergleich zu einem salzempfindlichen (RC222) und einem salztoleranten (FL478) Reiskultivar gemessen wurde. *cpm2* und *hebiba* verhielten sich wie das salztolerante Kultivar und opferten ältere Blätter durch erhöhte Aufnahme von Na^+ in diese, wodurch weniger Na^+ in die jungen Blätter aufgenommen wurde. Andererseits verhielt sich der Wildtyp wie das salzempfindliche Kultivar, welches eine erhöhte Aufnahme von Na^+ in allen Blättern zeigte. Der Wildtyp zeigte auch eine erhöhte Konzentration von JA und JA-Ile im Blatt, womit ein direkter Zusammenhang zwischen Na^+-Aufnahme und Jasmonat gezeigt wurden konnte.

Alle diese Ergebnisse über Jasmonat-regulatorische Systeme, welche Erkenntnisse über Stresswahrnehmung und Phytohormonwechselwirkung beinhalten, haben Licht in das Wissen über die Schnittstelle zwischen Signalleitung und Stressadaption gebracht.

ABSTRACT

Rice (*Oryza sativa* L.) is the staple food for more than half of the world population and salinity is one of the abiotic stress affecting severely on the crop productivity and on agriculture suitable land leading to strong social and economic impact. The plant hormones jasmonates which are a collection of jasmonic acids and its metabolites act as signaling molecules which play a vital role in biotic and abiotic stress tolerance and also development in plants. Here, we tried to find an innovative strategy to show how suppression of jasmonate signaling under salt stress can contribute to alteration in jasmonate sensitivity and salt stress response negating the detrimental effect of jasmonate insensitivity like decrease in fertility, susceptibility to pathogens etc.

In order to prove this strategy, we generated and analyzed transgenic rice plants and transgenic BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) suspension cells which expressed full and C terminal truncated (lacking Jas domain) OsJAZ8 protein under control of the salt inducible promoters of *ZOS3-11* and *ZOS3-12*. Using the transgenic tobacco, we were able to monitor the activity of the promoters and show that the induction of *OsJAZ8* gene led to jasmonate insensitive phenotype by showing reduced cell mortality, increase in cell length and decrease in cell cycle duration thereby show better performance especially under MeJA treatment, but also under salt stress. Interestingly, suppression of jasmonate signaling also resulted in an increase in auxin responsiveness in transgenic BY2 cell lines compared to the wild type showing that jasmonate acts as a modulator of auxin responsiveness.

In rice, transgenic seedling showed better tolerance to salt stress, which was in correlation with the increased amount of *OsJAZ8* transcript in ZOS3-11::JAZ8 and *OsJAZ8ΔC* in ZOS3-11:: JAZ8ΔC under salt stress compared to the wild type rice. The transgenic rice did not show any phenotypical difference under control condition but *OsJAZ8* and *OsJAZ8ΔC* contents were found to be high compared to the wild type.

In the second part of the study, characterization of ZOS3-11 and ZOS3-12 protein was done. These are C2H2 type zinc finger transcription factor homologous to STZ of *Arabidopsis* and there transcript expressions were highly induced in salt stress in a jasmonate-dependent

manner. Transient expression analysis using synthetic green fluorescent protein fusion proteins indicated that these transcription factors are preferentially localized to the nucleus even though it was lacking nuclear localization domain and binds to A(C/G)T sequence and had repression activity.

More evidences were obtained in addition to the previous study of showing better salt stress tolerance in the jasmonate biosynthesis mutants (*cpm2* and *hebiba)* compared to wild type by performing a detailed time course study to analyze the sodium and potassium uptake under salt stress with a salt sensitive (RC222) and salt tolerant (FL478) variety as control. The *cpm2* and *hebiba* behaved similarly to salt tolerant variety sacrificing its older leaf by an increase in uptake and accumulation of Na^+ in the older leaf and less accumulation of Na^+ in the younger leaves. On the other hand, the WT behaved similar to salt sensitive variety having increase in Na^+ uptake and distributed accumulation in all the leaves. The WT also showed increased accumulation of JA and JA-Ile in leaf with time showing a direct relation of jasmonate with increased uptake of Na^+

All these results obtained about jasmonate regulatory systems that link stress sensing and phytohormone crosstalk have shed light on the interfaces of signaling and adaptation to stress conditions.

1 INTRODUCTION

1.1 Stress and plants

Being sessile, plants are frequently exposed to unfavorable environmental conditions. These stress factors fall in two groups; biotic and abiotic. Biotic stress refers to biological factors such as insects, weeds, and pathogens (bacteria, fungi, viruses, nematodes)(Pinheiro&Chaves, 2011). On the other hand, abiotic stresses such as salinity, drought, low or high temperature, heavy metal exposure, high light levels are some of the main threats affecting plant growth and production. It is estimated that 90% of arable land is exposed to abiotic stresses (dos Reis *et al.*, 2012) and up to 70% of plant growth and productivity is impacted (Mantri *et al.*, 2012). Along with the mounting population pressure that we are facing globally and in order to feed the growing millions, we need to reduce the losses, even if the agricultural area cannot be increased. To achieve this, we must overcome the losses caused by abiotic stresses by gaining more understanding of the molecular mechanisms that underpin plant responses to abiotic stress.

1.2 Major abiotic stress: Salinity

Salinity, one of the major abiotic stresses limiting the crop production worldwide affects both wet and dry parts of the world and often referred to as “Silent Killer” of natural production, as it is the most deleterious factor killing plants and soil organisms when affected. Considering plants, salinity is defined as excessive concentrations of soluble salts in soils such as Ca^{2+}, Mg^{2+}, Na^{+} and anions SO_4^{2-}, Cl^{-}, HCO_3^{-} with exceptional amounts of K^{+}, CO_3^{2-}, and NO_3^{-} (Richards, 1954) and if the salt concentration is high enough to lower the water potential, it is called as salt stress (Levitt, 1980). According to the USDA salinity laboratory, saline soil can be defined as soil having an EC (Electrical Conductivity) of 4 dS m-1 (decisiemens per meter), where 4 dS m-1 ≈ 40 mM NaCl or more (Chinnusamy, 2005; Kotuby-Amacher, 2000).

Statistics show that more than 830 million hectares (ha) of land is salt-affected globally (FAO 2008) (Figure 1.1) and approximately two million ha of land becomes uncultivable due to excessive salinity each year (Umali, 1993). Of the 230 million ha of the world's irrigated land, 45 million ha (20%) has been salt-affected and is degraded by salt every year according to a study by Economics of Salt-Induced Land Degradation and Restoration. This is basically because of the higher sea levels bringing saline water further inland and exposing more crop growing areas to salty condition and also excessive use of irrigation water with improper drainage coupled with the use of poor-quality irrigation water. Based on the origin, soil salinity can be classified as primary salinity caused naturally, whereas other is human-induced salinity called as secondary salinity. The main source of all salts in the soil is the weathering of primary minerals in the exposed layer of the earth's crust as well as seawater since it contains around 500 mol m^{-3} NaCl (Taiz&Zeiger, 2002). When growing on salt-affected soils, crops must compete with salts in the soils for water as well as to cope with ion toxification, nutritional disorders and poor soil physical conditions to survive, therefore, reduction in total yield (Munns&Tester, 2008; Shrivastava&Kumar, 2015). By 2050, the world's population is predicted to reach 9.6 billion people and food production needs to increase approximately 70% by 2050 or 44 million metric tons annually to provide sufficient food for this population (FAO, 2009, 2011). This is a challenge because there is very little potential for future expansion of arable lands, whereas environmental stresses affecting crop production is increasing (Eckardt *et al.*, 2009; Tester&Davenport, 2003) To help sustain the increasing population, crops with enhanced salinity tolerance must be developed to increase productivity on salt-affected lands.

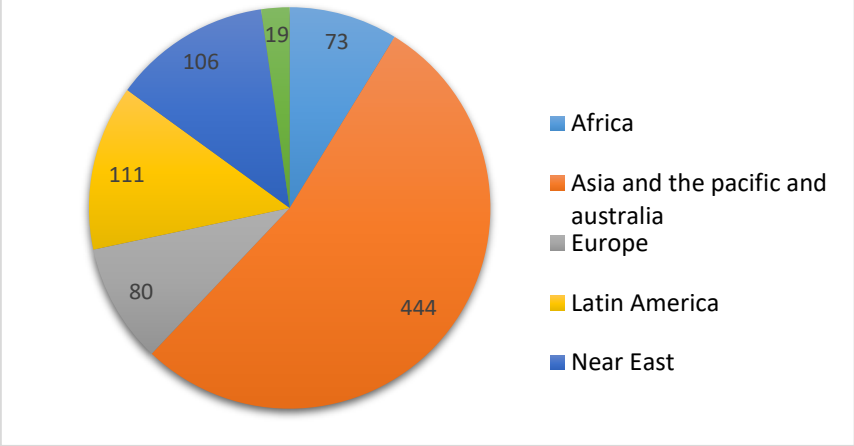


Figure 1.1: Global distribution of salt-affected soil (million ha)

Based on the level of salinity tolerance, plants can be divided into two groups—halophyte (salt plants) or glycophyte (sugar plants). Halophytes are plants that tolerate relatively high salt concentrations (400 mM NaCl), while glycophytes tolerate low concentrations (Flowers, 1977). However, at salt concentrations (20 mM to 200 mM) there is an overlap of growth responses between the two groups Halophytes accumulate excess Na^+ under saline conditions (Flowers&Yeo, 1981) and exhibit certain morphological and anatomical features like leaf succulence, thickening of cuticle, increased lignification, changes in number and diameter of xylem vessels and presence of salt glands which are effective devices for the secretion of salt from the plant body (Helder, 1956; Scholander, 1968).

; Scholander, 1968). Almost 80% of the Na^+ absorbed is accumulated in the leaves (Hall *et al.*, 1978). and to counteract this, halophytes must have an internal tolerance of NaCl level which is normally toxic to the non-halophytes (Maathuis *et al.*, 1992). Glycophytes, on the other hand, tend to exclude salt from leaves but may accumulate elevated levels of it in their roots and stems (Flowers&Yeo, 1981). Older leaves may accumulate more than younger leaves and buds. Most crops are glycophytes and their growth is retarded during salinity stress. Among the cereals, rye (*Secale cereale*) is the most tolerant (threshold of 11 dSm−1) and rice is the most sensitive crop plant with a threshold of 3 dSm−1 for most cultivated varieties (Brown, 2016).

1.3 How salinity affect plants?

Plants are affected by high salinity in two main phases, osmotic phase and ionic phase. To describe these a two-phase model of salt stress was proposed by (Munns *et al.*, 1995) (Figure 1.2). It showed timescale in days or weeks or months, depending on the species and the salinity level. During the first phase, the reduced soil water potential due to high concentrations of salts in the soil thermodynamically hamper the capacity of roots to absorb water owing to osmotic effect. This will cause a water deficit signal to be rapidly transmitted from roots to shoots and causes intracellular turgor reduction and decreased cell expansion. This osmotically caused loss of turgidity will affect membrane tension and perceived through changes in mechanosensitive ion channel activity by causing Ca^{2+} influx or Ca^{2+} burst.

In second phase, high concentrations of salts (Na^+ and/or Cl^-) within the plant becomes toxic resulting in an inhibition of many physiological and biochemical processes such as nutrient uptake and assimilation leading to ionic effect (Hasegawa *et al.*, 2000; Munns, 2002; Munns *et al.*, 1995; Munns&Tester, 2008). This happens especially in the older leaves, inducing tissue necrosis and early leaf senescence.

At the cellular and molecular levels, ionic toxicity also causes membrane disorganization and metabolic toxicity leading to Ca^{2+} influx which can be transduced through calcium dependent kinases into activation of the NADPH oxidase RboH generating ROS, apoplastic singlet oxygen, such that calcium influx is followed (with some delay) by a transient oxidative burst leading to oxidative stress (Dubiella *et al.*, 2013). This signal may promote the biosynthesis of various phytohormones and proteins leading to lowering of stomatal conductance which causes a decrease in carbon assimilation, inhibit photosynthesis, and reduce nutrient acquisition (Hasegawa *et al.*, 2000; Tuteja, 2007; Zhu, 2002), biomass production and ultimately yield (Munns, 2005; Munns&Tester, 2008; Roy *et al.*, 2014). Together, these effects reduce plant growth, development and survival. Sensitivity of a plant to salinity differ in the rate at which salt reaches toxic levels in leaves.

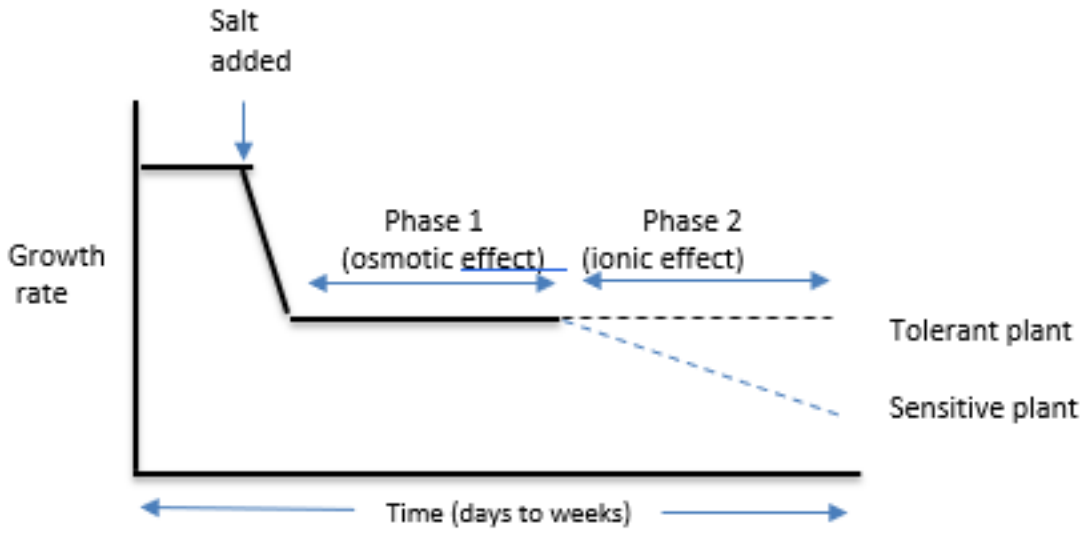


Figure 1.2: Schematic illustration of the two-phase growth response to salinity (Munns, 2005)

Another effect during second phase is ion imbalance. Due to similarity in physicochemical properties between Na^+ and K^+ (ie., ionic radius and ion hydration energy), Na^+ competes with K^+ for the metabolic processes like enzymatic reactions, protein synthesis and ribosomal functions for which K^+ plays a very important and it should be maintained within 100-200 mM range in the cytosol (Cuin *et al*., 2003; Walker *et al*., 1995). Na^+ inhibits the enzyme activity and leads to severe metabolism impairment inside the cells both in root and shoot (Marschner, 1995). It is important to have high cytosolic K^+/Na^+ ratio in the cytoplasm to prevent imbalances in the enzymatic reactions and to sustain plant survival under salt stress. So, control in accumulation of Na^+ in shoot has been correlated with salt stress tolerance in rice (Lutts, 1996) and maize (*Zea mays* L.) (Tester&Davenport, 2003).

1.4 Rice - its importance and threat by salinity

Rice (Oryza sativa L.) is a monocotyledonous angiosperm originally cultivated in tropical Asia, from 5000 years, later extended to temperate regions. Rice (*Oryza sativa* L) belongs to the family Poaceae (Gramineae), subfamily Bamboosoideae and tribe oryzeae. The *Oryza* genus has more than 20 species, of which two—*Oryza sativa* L. and *O. glaberrima*—are cultivated. *Oryza sativa* (Asian rice) is grown worldwide, with its two eco-geographic races *indica*, adapted to the tropics, and *japonica*, adapted to temperate regions and tropical uplands, cultivated in South-east Asian countries and Japan. *Oryza sativa* has many ecotypes or cultivars adapted to various environmental conditions. It is grown in all continents except Antarctica. *Oryza glaberrima* (African rice) is grown and cultivated in parts of West Africa (Linares, 2002; Vaughan *et al*., 2008).

The basic chromosome number of rice is n=12. The species can be either diploid or tetraploid. In this respect, *Oryza sativa* L. and *Oryza glaberrima* L both are diploid species (2n= 24). *Oryza sativa* L. is the first fully sequenced crop genome and is a model crop species.

Rice is one of the most important and staple food for half of the world's population and hence has an exceptional agricultural importance. Rice is cultivated in 114 of the 193 countries over the world in six continents, Asia, Africa, Australia, Europe, Latin America and North America (Virmani&llyas-Ahmed, 2008) where its cultivation covers a global area of 156 million

hectares of land producing 650 million tons of crop. In humid and sub-humid Asia, rice is the single most important source of employment and income for rural people, and it provides 50%–80% of the calories consumed by more than three billion Asians (Hossain, 1995 ; Khush, 2005). It yields about one third of the total carbohydrate source. It provides the considerable amount of recommended Zinc and Niacin (Munns, 2005). Rice protein is biologically richest as its digestibility is very high (88%). It is the second most important crop in the world after wheat, covering almost 90% of area across Asia alone. Main rice producing countries are China, India, Indonesia, Bangladesh, Vietnam, Myanmar, Thailand and Philippines (FAO, 2011) (Figure 1.3). The use of the crop varies widely ranging from its use as food in cereals, snacks, brewed beverages, flour, rice bran oil to its use in religious events across India. The medicinal value of the crop adds on more to the list.

Rice is glycophyte and salinity interferes with its growth and development, plant adaptation and stress responses. It is salt susceptible cereal, especially, at its early vegetative and later reproductive stages (Maas&Hoffman, 1977; Shannon *et al.,* 1998; Todaka *et al.,* 2012). Salinity stress affects seed germination, seedling growth, leaf size, shoot and root length, shoot dry weight and fresh weight, number of tillers per plant, flowering stage, spikelet number and productivity (Hakim *et al.,* 2010; Moradi&Ismail, 2007; Munns&Tester, 2008; Zeng&Shannon, 2000). Growth differences among various genotypes in response to salinity are dependent on the salt concentration and the degree of salt tolerance (Eynard *et al.*, 2005) and also due to additive gene effect (Sahi *et al.,* 2006). Among 180,000 rice genotypes screened by International Rice Research Institute (IRRI, 2013), 17% has acceptable salt tolerance at an electrical conductivity EC of 10 dS/m at seedling stage (Gregorio *et al.,* 2002). Apart from seedling stage, flowering stage is another highly sensitive growth stage which is affected by salinity stress though salt tolerance at seedling stage is independent of flowering/reproductive stage (Singh *et al.*, 2004). Even at EC as low as 3.5 dSm−1, rice loses about 10% of its yield, and 50% yield loss was recorded for rice at EC7.2 dSm−1 (Umali, 1993).

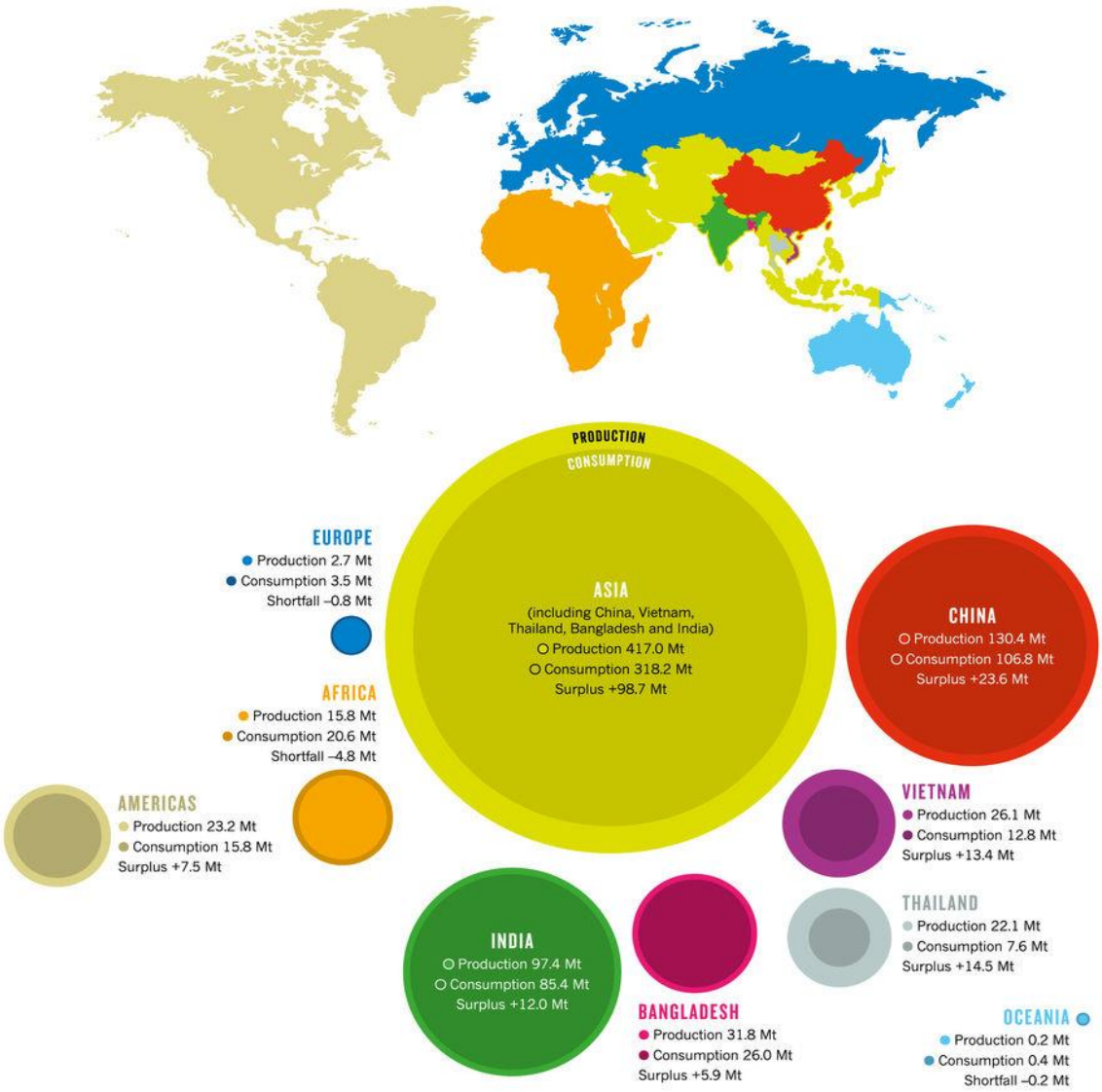


Figure 1.3: Statistics of Rice production worldwide (FAO, 2011)

1.5 Mechanism of salt tolerance in rice

Plants have evolved various mechanisms to acclimatize to salt stress. In general salt tolerance mechanism can be categorized into a) Na^+ exclusion by selective ion uptake of Na^+ at molecular level, b) osmotic tolerance via osmotic adjustment, c) tissue tolerance to Na^+ or Cl^- via compartmentation of the accumulated toxic ions in the vacuole, synthesis of compatible

solutes and production of enzymes catalyzing detoxification of reactive oxygen species (Munns&Tester, 2008; Roy *et al.,* 2014) (Figure 1.4).

a) Ion exclusion and tissue tolerance: Root epidermis including root hairs are primary sites for Na^+ uptake and are mainly involved in Na^+ and Cl^- transport processes in roots, which prevent the excess accumulation of Na^+ and Cl^- in leaves and includes retrieval of Na^+ from the xylem, and efflux of ions back to the soil.

In rice plants, Na^+ transport of salts happens by selective uptake by root cells through symplastic and apoplastic routes (Das *et al.,* 2015). Rice plants protect younger leaves from ion injury by accumulating toxic ions like Na^+, Cl^- and NO_3^- in older leaves. There are ion pumps like H^+ pump ATPase, Na^+ transporter *(OsHKT1)*, Na^+/H^+ symporters (*OsHKT2)*, Na^+/H^+ antiporters (*OsSOS1*, *OsNHX1), K^+ inward rectifying channel (OsAKT1)* help in Na^+ transport and compartmentation maintaining cell ion homeostasis (Jeschke, 1984). Salinity leads to up-regulation of *OsHKT1;1*, *OsHAK10* and *OsHAK16* advances the accumulation of Na^+ in old leaves, and increased *OsNHX1* expression contributes to Na^+ compartmentalization vacuoles of old leaves (Wang *et al.,* 2012). One endosomal Na^+/H^+ antiporter (OsNHX5) and four vacuolar Na^+/H^+ antiporters (OsNHX1, OsNHX2, OsNHX3 and OsNHX4) in rice were reported (Bassil *et al.,* 2012). Root cap cells helps in Na^+ exclusion and Na^+ could enter cells through high affinity K+ carriers (HAK) or through low affinity channels called nonselective cation channels (NSCC) that are strongly influenced by Ca^{2+}.The Na^+/H^+ exchange in vacuoles is determined through vacuolar H^+-ATPase and vacuolar H^+- translocating pyrophosphatase (Blumwald&Poole, 1987) which increases uptake of Na^+ to vacuoles and facilitate enhanced storage of Na^+ conferring to higher tolerance by reducing Na^+ in cytosol**.** Recently a calcium channel OSCA1 from *Arabidopsis thaliana* that is gated by hyperosmotic stress was discovered (Yuan *et al.,* 2014) which might be a central player for the perception of osmotic challenge of the membrane.

Another pathway known as salt overly sensitive (SOS) pathway where SOS3 (Ca^{2+} binding protein) senses the cytosolic calcium ion signal elicited by salt stress directly interacts and activates SOS2, a serine/threonine protein kinase forming SOS2-SOS3 complex which

phosphorylates SOS1, a Na^+/H^+ antiporter on cell membrane, which extrudes Na^+ out of the cell and maintaining Na^+ homeostasis and salt tolerance (Guo *et al.,* 2004. ; Quintero *et al.,* 2002). From various results, it can be concluded that the change in $[Ca^{2+}]$cyt is not uniform and varies with species, cell type or tissue type (Cramer&Jones, 1996). Specific Ca^{2+} signatures are also important for plant cells to follow the subsequent events in the signalling process. A comparison of two grapevine cell lines that differ in salt tolerance revealed that efficient adaptation was correlated with a more rapid uptake of sodium into the cytoplasm indicating that the concomitant increase of cytosolic calcium and sodium might act as a signal triggering salinity adaptation (Ismail *et al.,* 2014a). The adaptive salt overly sensitive (SOS) module cannot only extrude sodium from the cytoplasm, but also links cytosolic sodium with calcium signaling (Ismail *et al.,* 2014b).

b) Osmotic tolerance: This is accomplished via accumulation of high concentration of inorganic ions or low molecular weight organic solutes like sugars, organic acids, polyols. They also include nitrogen containing compounds such as amino acids, amides, imino acids, proteins and quaternary ammonium compounds. It is regulated by long distance signals that reduce shoot growth and is triggered before shoot Na^+ accumulation, it involves the plant's ability to tolerate the drought aspect of salinity stress and to maintain leaf expansion and stomatal conductance (Rajendran *et al.,* 2009). Reports shows that proline plays an active role in osmotic adjustments shielding the enzymes and membranes, also providing energy and nitrogen for utilization during exposure to salinity (Cram, 1976; Perez *et al.,* 1993). The toxic effects of salinity are masked by accumulation of glycine and betaine in rice (Pareek *et al.,* 1997). They balance the cellular pH, detoxify the cells and scavenge the ROS species. Studies show that a signalling pathway similar to that of yeast MAPK–HOG pathway may be involved in regulation of osmolyte biosynthesis in plants. In Arabidopsis, high osmolarity inactivates osmosenser AtHK1 leading to accumulation of active non-phosphorylated response regulator which in turn activates osmolyte biosynthesis by activating MAPK pathway and on the other hand at low osmolarity, active form AtHK1 inactivates a response regulator by phosphorylation. A significant increase in soluble protein content and positive correlation has been ensured in tolerant rice seedlings compared to the sensitive ones.

Generally, most rice varieties are sensitive to salinity, however, some traditional *indica* rice varieties such as Pokkali, Nona Bokra and Kala-rata are fairly tolerant to salinity (Yeo *et al.*, 1990).

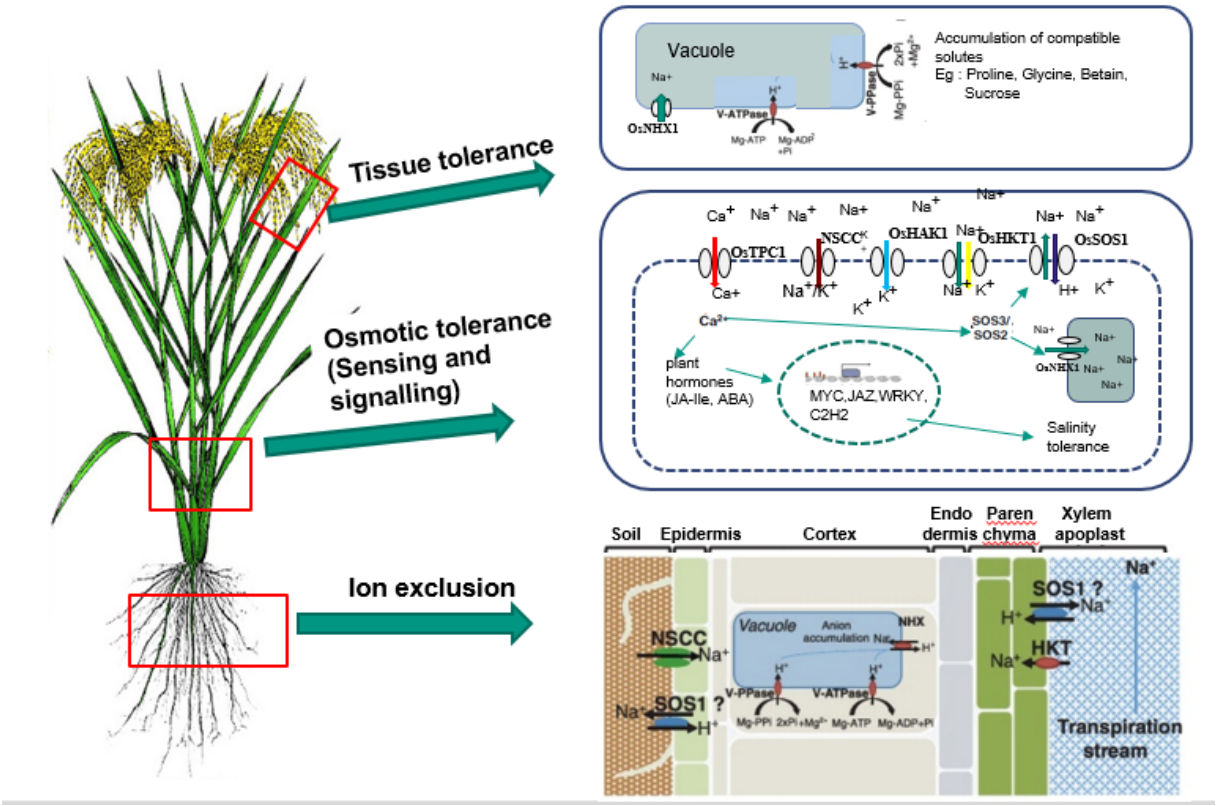


Figure 1.4: Schematic representation of overview of the plant responses under salt stress. (Some parts of figure adapted from Roy *et al.*,2014)

In addition to osmotic and ionic stress, alkalinity causes the precipitation of other ions like phosphates and metallic micronutrients which could destroys cellular structure of the roots (Li *et al.*, 2009). During alkaline conditions, the cell expansion mechanism is disturbed by interruption in slightly acidic pH of 5.5 by proton ATPases localized in the plasma membrane (Haruta *et al.*, 2010). The activity of osmotically induced calcium influx is expected to be impaired, because calcium enters the cell by cotransport with protons, as an effect, the superoxide anions generated by the NADPH oxidase RboH will not be dissipated due to the

absence of protons as electron acceptors, leading to an accentuated stress-induced oxidative burst. In addition to sequestering sodium in the vacuole, and quelling the accentuated oxidative burst, adaptation to alkalinity would also require powerful buffering of the apoplast, which might either be achieved through upregulation of proton ATPases or through secretion of organic acids.

The comparison of the three aspects of osmotic stress illustrates that each specific condition requires a specific adaptive response, which seems to be determined by specific equilibriabetween different stress inputs. For instance, stomatal closure will reduce water loss by transpiration, but it will also reduce photosynthetic efficiency and lead to secondary photooxidative stress caused by unbuffered electron transport in the thylakoid (Pinheiro&Chaves, 2011). Similarly, the synthesis of polyamines binds precious bioavailable nitrogen (Alcázar *et al.,* 2006). Therefore, these adaptive responses have to be carefully adjusted to growth and development. It is this adjustment, where phytohormonal signaling links with stress adaptation and among different phytohormones, the jasmonates and their signaling system seems to act as a hub, where different inputs are processed to yield an appropriate adaptive response.

1.6 How plant hormones help to adapt salt stress

Phytohormones are small molecules transported to regulate plant growth, development, and stress responses. Some plant hormones were identified during the first half of the twentieth century eg, auxin, abscisic acid (ABA), cytokinin (CK), gibberellin (GA) and ethylene (ET). Several additional compounds are meanwhile considered as hormones, including jasmonate (JAs), brassinosteroids (BRs), salicylic acid (SA), nitric oxide (NO), polyamines (PAs) and strigolactones (SLs) (Santner & Estelle, 2009). Among those plant hormones, ABA and Jasmonates (JAs) and its methyl esters (MeJAs) play central roles in plant adaptation to stress (Fonseca *et al.,* 2009; Gfeller *et al.,* 2010a; Koo *et al.,* 2009; Wasternack&Hause, 2013). Phytohormones help to deviate towards defense from plant metabolism e.g. by root growth and cell cycle inhibition, reduction of photosynthesis, transpiration and seed germination (Soares *et al.,* 2010), induction of stomatal closure (Suhita *et al.,* 2004), senescence (Seltmann *et al.,*

2010) upregulation of antioxidant enzymes, elicitors of plant secondary metabolism (De Geyter *et al.,* 2012)and many other examples.

1.6.1 Debatable role of Jasmonates in salinity tolerance

Jasmonates are one of the important phytohormones involved in plant defense responses against biotic stress such as wounding by insects and pathogens attack (Wasternack&Parthier, 1997) as well as abiotic stress like salt and water stress (Creelman&Mullet, 1995; Moons *et al.,* 1997; Pedranzani *et al.,* 2003; Tani *et al.,* 2008).

In our study, jasmonates and its signaling will be on prime focus as increasing evidence have been found that jasmonates (JA) plays a vital role in abiotic stress (Kazan, 2015; Riemann *et al.,* 2015) and reported to participate in the response to salinity stress (Moons *et al.,* 1997). However, it is difficult to make a direct connection between JA levels and salt stress adaptation. In some cases, endogeneous JAs increased under high salt stress in rice roots (Moons *et al.,* 1997) and in tomato (Pedranzani *et al.,* 2003) and exogeneous JAs gained salt stress tolerance in soybean (Yoon *et al.,* 2009) and rice (Kang *et al.,* 2005), but in some other cases, when comparing two grapevine cell lines shows that the accumulation of JA and JA-Ile was more in the sensitive *Vitis riparia* when compared with the salt-tolerant *Vitis rupestris* (Ismail *et al.,* 2014a) and in case of barley segments (Kramell *et al.,* 2000) and rice seedlings (Takeuchi *et al.,* 2011) JA was induced in osmotic stress but not in salt stress. Recent reviews (Kazan, 2015; Riemann *et al.,* 2015) reported some correlation between high JA content and root growth inhibition as a sign of salt stress adaptation in *Arabidopsis* (Dong *et al.,* 2013) and rice (Toda *et al.,* 2013). Recently it was reported that suppression of OsJAZ9, a repressor of JA signalling, increased sensitivity to salt (Wu *et al.,* 2015). Conversely, another report showed that in jasmonic acid (JA) biosynthesis rice mutants *(cpm2 and hebiba*) impaired in the function of ALLENE OXIDE CYCLASE (AOC) (Riemann *et al.,* 2013) and *CYP94C2b* (genes encoding for an enzyme which metabolizes JA) overexpressing plants (Heitz *et al.,* 2012 ; Kitaoka *et al.,* 2011; Koo *et al.,* 2011; Kurotani *et al.,* 2015), decreased amounts of JA led to an improved performance on high concentrations of NaCl. An important study from our group could demonstrate that the jasmonate mutants (*cpm2* and *hebiba*) which performed better in salt stress accumulated less Na^+ ions in their leaves, elevated induction of antioxidants and absence

of JA precursor 12-OPDA (which also plays a role in destabilizing the redox homeostasis), correlated with an increased ROS-scavenging activity and higher activity of enzymes in the antioxidative pathway, such as glutathione S-transferase (Hazman *et al.*, 2015).

All these reports make us to think, what are the real reasons behind these discrepancies? Whether these reflect gaps in knowledge, comparing result in non-comparable conditions (different developmental stages and plant species) or fundamental differences in mechanism in different situation. How can plants behave so differently to similar situation? And how can one make a conclusion to solve this discrepancy which can lead us to design plants adaptable to salinity?

1.6.2 Fine tuning of jasmonate signalling leads to salt stress adaptation

In case of salinity, JAs production starts by the lipid peroxidation of the chloroplast membrane as a result of oxidative stress induced by salt stress. This production if not controlled will lead to cell death induction, which was proved in *Arabidopsis* protoplast (Zhang&Xing, 2008) and *Vitis* cell suspensions (Repka *et al.*, 2004) by MeJA treatment in a concentration- and time-dependent manner, and in the absence of JA, a block of PCD (programmed cell death) and the activation of defense and detoxification mechanisms was observed due to maintenance of low levels of lipid peroxidation limiting oxidative damage (Ramel *et al.*, 2013). All these reports are showing that jasmonate can act as decision maker between cell death and adaptation based on the ability of the cells to respond and control the jasmonate production and signaling. In a recent review (Ismail *et al.*, 2014b) it was emphasized on the fact that earlier deactivation of JA signaling pathways seems to be beneficial for the salt adaptation rather than delayed deactivation of the sustained JA signaling leading to destructive effects and sensitive phenotype. So, it is solely not the presence or absence of JAs alone that decides the response to salinity as adaptive or destructive, but temporal signature, amplitude of the jasmonate signaling and also cross-talk with other signaling pathways (activating or shifting to different hormonal signaling like ABA also improves adaptation).

1.6.3 How jasmonate signaling is deactivated and why is it important?

The transient action of JA production in the plant indicates that there are mechanisms to switch off the signaling to avoid unfavorable effects of overshooting JA signaling. Inactivation of JA signaling takes place majorly at two different steps. First, in response to JA signaling, JAZ repressors which are amongst the first genes to be induced provides a negative feedback loop that aims at switching off JA responses rapidly. The newly synthesized JAZ proteins interact with and restore the repression of MYC2, which in turn deactivates the JA signal transduction pathway (Chini *et al.*, 2007; Thines *et al.*, 2007; reviewed by Wager and Browse, 2012) (more details in section 1.6.3). Secondly, inactivation of the bioactive JA-Ile can be achieved either by hydroxylation (12-OH-JA-Ile) and/or carboxylation (12-COOH-JA-Ile) of JA-Ile (Koo *et al.*, 2011; Kitaoka *et al*., 2011) which are mediated by Cytochrome P450 enzymes (CYP94B1, B3, and C1) (Heitz *et al.*, 2012 ; Koo *et al.*, 2011; Van Doorn *et al.*, 2011). This pathway is known as ω- oxidation pathway, is now recognized as a major route for catabolism of the hormone. In some previous wounding studies in *Arabidopsis*, catabolism of JA-Ile in 12-OH-JA-Ile and 12-COOH-JA-Ile occurs relatively fast (~90 min) after wounding of leaf (Glauser *et al.*, 2008) and the enzymes seemed to be induced by wounding and jasmonates (Kandel *et al.*, 2007). Inactivation of JA-Ile also occurred by the hydrolysis of Ile from JA mediated by two amidohydrolases in *Arabidopsis*, ILL6 and IAR3, which also are involved to regulate plant defense to herbivores (Widemann *et al.*, 2013; Woldemariam *et al.*, 2012).

Taking all these points into consideration, one of the main idea in this PhD thesis focuses on the aspect to find a strategy which can be adopted to improve salt tolerance in rice by modifying the temporal expression of jasmonate and its signaling without causing negative effect of jasmonate deficiency on the plant.

1.6.4 JA biosynthesis and mode of action

In order to modify hormonal responses, it is important to know how JA biosynthesis and signaling operates. They are interlinked (Figure 1.5) by a positive feedback loop whereby jasmonates stimulate their own synthesis (Sasaki *et al.*, 2001). The extent of activity of this positive feedback loop appears to differ in tissues near a site of stress where there is strong

feedback leading to the production of high levels of JA, and distal tissues where JA levels increase but remain relatively low (Glauser *et al.*, 2009). Given below are the steps of JA synthesis

Step 1: Initiation of JA synthesis takes place in plastids. Lipoxygenases (LOXs) oxygenate triunsaturated fatty acids (18:3) to produce 13-hydroperoxyfatty acids. These are substrates for allene oxide synthase (AOS) which generates unstable allene oxides that are cyclized by allene oxide cyclase (AOC). The resulting compound, 12-oxo-phytodienoic acid (12-OPDA) is exported to the peroxisome.

Step 2: JA synthesis completion in peroxisomes (Figure 1.5). First, 12-OPDA is reduced from cyclopentenones to cyclopentanones by the peroxisomal enzyme OPDA reductase (OPR). The resulting compound OPC8, is then subject to β-oxidation (3 rounds for OPC8) to produce JA. JA is exported by an unknown mechanism to the cytosol where it is conjugated specifically to isoleucine (Ile) by enzyme JAR1 (Staswick&Tiryaki, 2004)**.** It presumably diffuses into the nucleus where it binds COI1–JAZ receptor complexes to activate jasmonate signaling pathway (explained below).

Step 3: Biochemical diversification.: Both JA and JA-Ile undergo diverse modifications *in vivo*. For example, formation of JA-glucose esters (Swiatek *et al.*, 2004), 12-hydroxy-JA (12-OH-JA) and its sulfated and glycosylated derivatives (Gldda *et al.*, 2003; Miersch *et al.*, 2008), volatile methyl-JA (MeJA), and JA-amino acid conjugates other than JA-Ile (Wang *et al.*, 2007). The ω-oxidation pathway, in which JA-Ile is converted to 12-hydroxy-JA-Ile (12-OH-JA-Ile) and then further oxidized to dicarboxy-JA-Ile (12-COOH-JA-Ile) (refer section 1.6.3 for details).

Step 4: Signaling.: JA signaling involves hormone induced degradation of a transcriptional repressor (Figure 1.6). In JA signaling, the repressor is the jasmonate ZIM/TIFY-domain (JAZ/TIFY) proteins known as JAZ proteins. In response to elevated JA levels due to stimulation by various stress factors, JAZ proteins are degraded in an SCF (for SKP1-CUL1-F-box)-type ubiquitin ligase SCF^{COI1}-dependent manner via the 26S proteasome, and this leads to the rapid activation of JA responses, such as the expression of JA-responsive genes (Chini *et al.*, 2007; Pauwels&Goossens, 2011) (Pauwels *et al*.,2011; Chini *et al*., 2007; Thines *et*

al.,2007). JA signaling requires the biologically active conjugate of JA with the amino acid Ile which is synthesized from the inactive JA, by JAR1 (see above, Staswick & Tiryaki.,2004). In the absence of JA-Ile, JAZ proteins which form homo- or heterodimers repress the transcriptional activity and turn off the expression of the early JA-responsive genes by binding to bHLH transcription factors (e.g. MYC2, MYC3, MYC4 and MYC5) that are activators of JA responses. JAZ proteins also recruit the general co-repressors TOPLESS (TPL) and TPL-related (TPR) proteins through an interaction with the adaptor protein Novel Interactor of JAZ (NINJA) or sometimes directly. Immediately after destabilizing JAZ by the action of JA-Ile, transcription factors such as MYC2, MYC3 and MYC4 are released from repression activating early JA responses. After JA responses are switched on, hormone signaling is attenuated by induction of the JA-responsive *JAZ* genes to avoid the inhibitory effect that over-activation of JA responses has on plant growth (Cheng *et al.,* 2011; Fernández-Calvo *et al.,* 2011; Figueroa&Browse, 2012; Niu *et al.,* 2011).

Step 5: Production and targeting of JA synthesis enzymes. 13-Lipoxygenases (13-LOXs) are synthesized on cytosolic ribosomes and targeted to the plastid. The same is true of Allene oxide synthase (AOS) and Allene oxide cyclase (AOC), Oxophytodienoic acid reductaxse3 (OPR3) is also encoded by a nuclear gene and is sent into the peroxisome (Schaller&Stintzi, 2009).

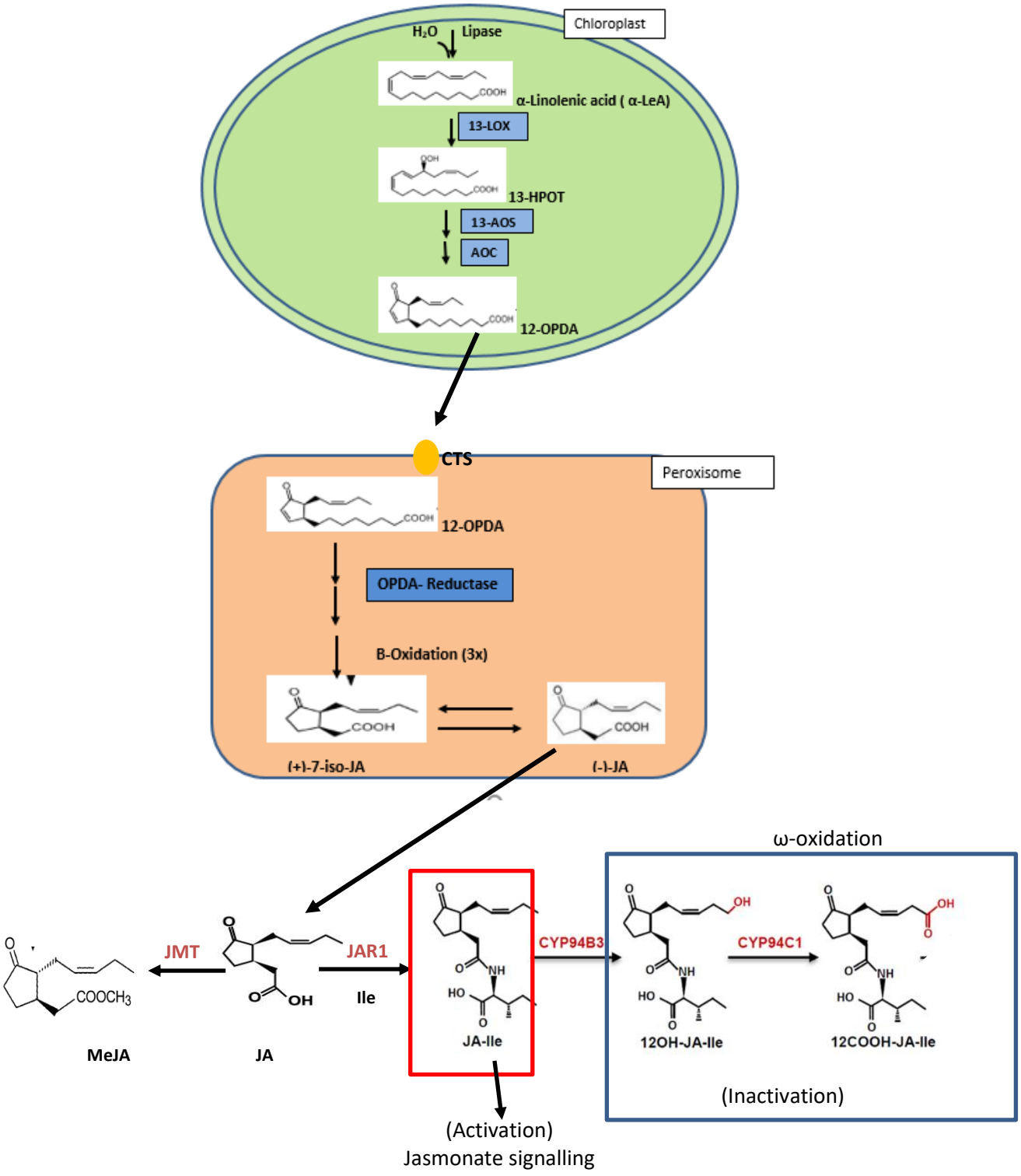

Chloroplast
H₂O
Lipase
α-Linolenic acid (α-LeA)
13-LOX
13-HPOT
13-AOS
AOC
12-OPDA
CTS
Peroxisome
12-OPDA
OPDA- Reductase
B-Oxidation (3x)
(+)-7-iso-JA
(-)-JA
ω-oxidation
JMT
JAR1
Ile
CYP94B3
CYP94C1
MeJA
JA
JA-Ile
12OH-JA-Ile
12COOH-JA-Ile
(Inactivation)
(Activation)
Jasmonate signalling

Figure 1.5: Biosynthesis and enzymatic modifications of jasmonic acid. The JA biosynthesis takes place in the two organelles chloroplast (green) and peroxisome (orange). It initiates in the plastid from membrane lipids by lipases to release 12-OPDA with series of intermediates and the enzymes involved (blue boxes). The import into OPDA into the peroxisome takes place via the ABC transporter CTS (yellow) (Theodoulou *et al.*, 2005). From peroxisome the jasmonic acid is released into the cytosol and modified to JA-Ile which is oxidized/inactivated to 12-OH-JA-Ile and 12-COOH-JA-Ile. Some important jasmonic acid modifications are shown. Abbreviations: 13-LOX: 13-Lipoxygenase, 13-HPOT: 13-Hydroperoxylinolenic Acid, AOS: Allen oxide synthase, AOC: Allen oxide cyclase, OPDA: 12-oxo-Phytodienoic acid, OPR: OPDA-Reductase, CTS: COMATOSE, JA: Jasmonic acid, MeJA: Methyl jasmonate, JAR1: JASMONATE RESISTANT 1, JMT: JA-. Methyltransferase, 12-OH-JA-Ile: 12-hydroxy-JA-Ile and 12-COOH-JA-Ile: dicarboxy-JA-Ile. (Figure modified from; (Dhakarey *et al.*, 2016)

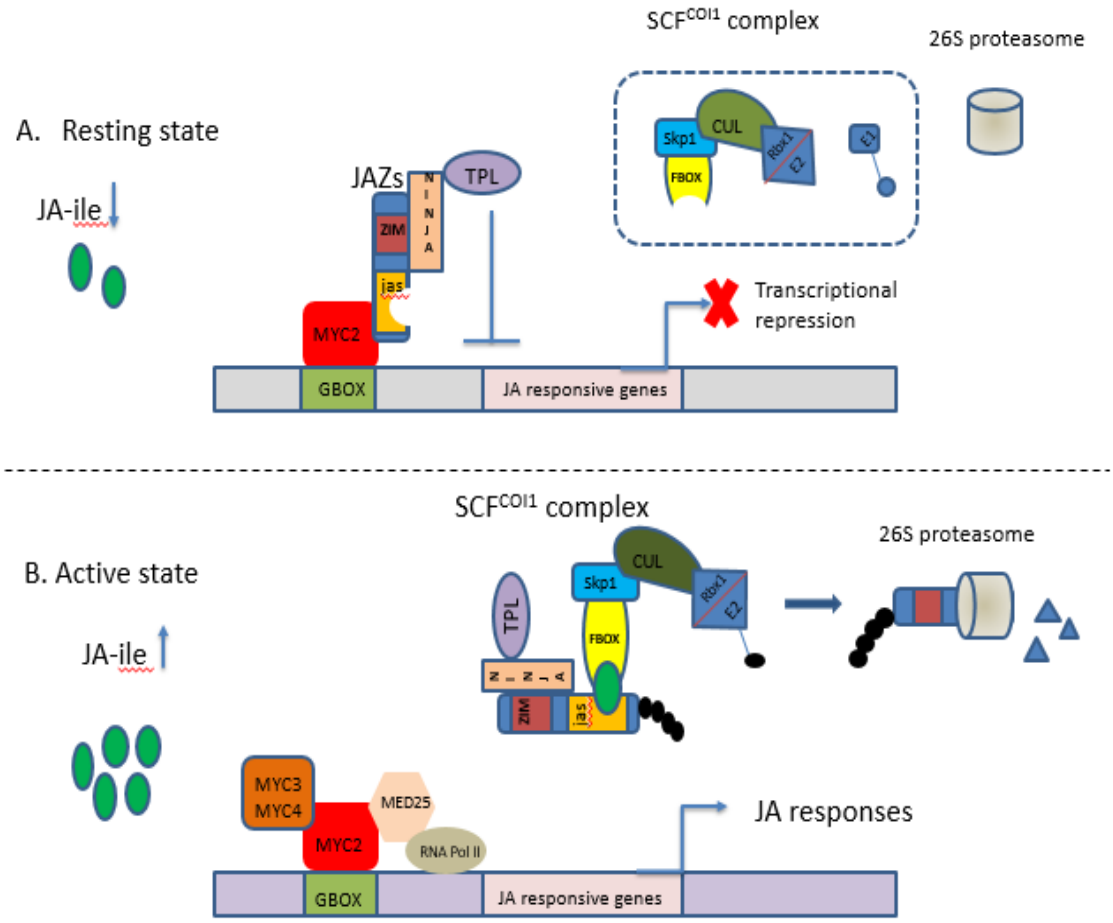


Figure 1.6: Jasmonate biosynthesis and signaling A) In the resting state, when little JA-Ile is present, MYC2 is bound to the G-box within the promoter region of the JA response genes. The transcription is inactivated by binding the JAZ repressors to MYC2. In addition, the Corepressor TPL is bound to the JAZ proteins via the NINJA adapter protein. (B) After stimulation by JA-Ile, the JAZ repressors are bound to the SCF complex, ubiquitinated (black spheres) and degraded in the 26S proteasome. This liberates MYC2 from the repression complex and the transcription of the JA response genes can begin. Abbreviations: JA-Ile: jasmonic acid isoleucine, MYC2, MYC3, MYC4: bHLH zip transcription factor, MED25: Mediator of RNA polymerase II transcription subunit 25, JAZ: JASMONATE ZIM DOMAIN, NINJA: Novel of Interactor of JAZ, TPL: TOPLESS, SCF: Skp1 / Cullin / F box complex , COI1: CORONATINE INSENSITIVE 1.

1.6.5 What are JAZ proteins and importance of Jas domain?

It is obvious from the jasmonates triggered signaling cascades, (JASMONATE-ZIM DOMAIN) JAZ repressor protein play a significant role which act as negative regulators of the transcription of jasmonate-responsive genes (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2007). This action is probably not performed by directly binding gene promoter sequences because JAZ proteins lack recognizable DNA-binding domains. However, their interaction with the transcription factor MYC2 suggests that JAZ proteins control jasmonate-related gene expression by preventing the function of transcriptional activators but the exact mechanism is still not known. There are 15 JAZ proteins in rice which are located on six chromosomes. Five OsJAZ genes were present on chromosome 3, and two each on chromosome 4 and 7. One on chromosome 8, two on chromosome 9 and three on chromosome 10 (Thines *et al*., 2007). Overexpression of *OsJAZ9* led to an increased salt tolerance (Ye *et al.*, 2007), while its suppression reduced salt tolerance (Wu *et al.*, 2015), *OsJAZ8* was found as a JA-inducible gene (Yamada *et al.*, 2012). Overexpression of *OsJAZ10* caused increase in shoot growth and seed size (Hakata *et al.*, 2012), and *OsJAZ1* (*eg2*) mutants showed an altered floral development (Cai *et al.*, 2014). More studies have to be done to know function of more JAZ genes.

Going into the structure of JAZ protein. Basically, there are the three conserved regions or domains in JAZ proteins: Jas domain, ZIM domain (Figure 1.7). The N-terminal (NT) region

contains a weakly conserved domain which remains largely uncharacterized bur have some implication of protein-protein interactions (Hou *et al.,* 2011). The Jas domain is a conserved region towards the C-terminus of JAZ protein and constitute binding site of COI1 in the presence of JA-Ile leading to the timely JAZ protein degradation upon jasmonate perception (Chini *et al*., 2007; Thines *et al*., 2007; Melotto *et al*., 2008; Chung & Howe, 2009). This domain is also necessary for the interaction of JAZ proteins with MYC2 (Chini *et al*., 2007; Melotto *et al*., 2008; Chini *et al*., 2009). The ZIM domain sits around the center of the JAZ proteins and contains a conserved TIFY motif (TIFYXG). This domain mediates homo- and heteromeric interactions between many JAZ proteins, a capability that is critical for their function as negative regulators of jasmonate signaling (Chini *et al.,* 2009; Chung&Howe, 2009). These interactions may be necessary to allow proper assembly of JAZ proteins into nuclear bodies (Grunewald *et al.,* 2009) which may contain the macromolecular complexes regulating jasmonate responses. It is known now that the C-terminal Jas domain of the JAZ protein plays a key role in destabilizing JAZ repressors as several reports have shown that C-terminal truncated JAZ proteins (JAZΔC) are more stable in the presence of JA and shows JA-insensitive phenotypes. (Thines *et al*., 2007; Chini *et al*., 2007). This dominant action of JAZΔC is because the protein interacts and represses the activity of MYC2 but fails to interact with COI1 (Melotto *et al.,* 2008).

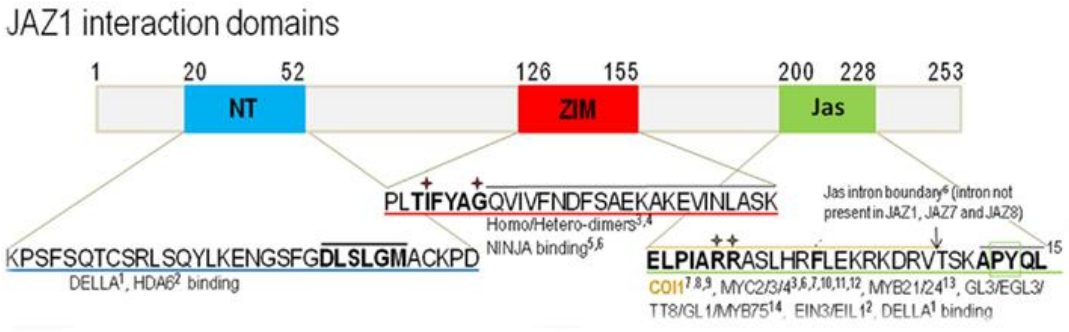


Figure 1.7: Schematic of JAZ1 interaction domains. Colored boxes indicate conserved domains with the sequence. The green box represents jas domain and the yellow bar indicates residues sufficient for COI1 binding and the green stars mark residues required for COI1 binding. Red box is the ZIM domain with TIFY region and the red stars mark residues required for forming homo- and/or heterodimers. The blue box is NT domain. And the black bars over bold residues mark a

conserved EAR-like motif. Abbreviation: NT-N-terminal, ZIM: ZINC-FINGER EXPRESSED IN INFLORESCENCE MERISTEM. (Figure adapted from (Wager&Browse, 2012).

1.6.6 Jasmonate crosstalk with other hormones

Recent advances have shown that hormones act through a network of interacting responses rather than through isolated linear path. Besides the role of Jasmonates in stress regulation, there are evidences of extensive interaction between jasmonates and other phytohormones like auxin (Kazan&Mannar, 2008). These interactions are mostly governed by shared components of signal transduction pathways (Ren *et al.,* 2005; Szemenyei *et al.,* 2008; Tiryaki&Staswick, 2002) or modulating synthesis and action of another (Hoffmann *et al.,* 2011). For example, JA-responsive genes have been reported to be repressed or induced by exogenous auxin (DeWald *et al.,* 1994; Tiryaki&Staswick, 2002). And there has been growing evidence showing the antagonistic effect of jasmonates and auxin regulating cell length and coleoptile photo and gravitropism (Gutierrez *et al.,* 2012; Gutjahr *et al.,* 2005; Riemann *et al.,* 2003; Sun *et al.,* 2011). Accumulation of IAA and upregulation of enzymes in IAA biosynthetic pathway was shown by cold and heat stress while the pattern was opposite under drought conditions. But JA level and JA responsive genes were increased upon cold and drought and suppressed upon heat treatment, implying different regulation of biosynthesis and signaling of JA and IAA upon diverse abiotic stresses. The underlying mechanisms as well as the biological context is far from understood, but one point of convergence might be the GH3 family of acyl acid amido synthetases which contribute to amino acid conjugation of both IAA and JA, which in case of JA generates the active signal, whereas it might be a mechanism of inactivation in case of IAA (Khan&Stone, 2007; Staswick *et al.,* 2005). And another common link is AXR1 which is a positive regulator of both SCF^{TIR1} and SCF^{COI1} which are the ubiquitin-proteosome pathway modulators for auxin and jasmonate signalling pathway required for the degradation of AUX/IAA and JAZ proteins. The *axr1* mutants shows a JA-insensitive phenotype in addition to resistance to auxin (Tiryaki & Staswick, 2002)

1.7 Role of C2H2 zinc finger transcription factors in salt stress response

Even though it is well established that plant hormones are critical players in adapting to abiotic stress, transcription factors mediate translation of hormonal signaling into defense gene activation. C2H2 (Cys2His2) proteins are one such group transcription factors existing as a superfamily which plays a key role in plant growth development, and defense responses.

1.7.1 C2H2 zinc finger transcription factors: structure and function

Cys-2/His-2-type zinc finger also known as classical or TFIIIA-type finger is one of the best characterized DNA-binding motifs found in eukaryotic transcription factors. It was first found in *Xenopus* oocytes (Miller *et al.,* 1985). In plants, these were first reported in petunia *(Petunia hybrida)* and was known as ZPTs and thus the C2H2-type ZFPs were also called ZPTs in plants. In plants mostly, the zinc finger proteins exist as a multiple fingered (one to four) where the adjacent fingers as separated by a long spacer that is highly variable in length and sequence from one protein to another. This is not the case with animal and yeast protein where in general contain a cluster of multiple fingers separated by a short spacer (6–8 amino acids) known as an HC-link (Klug&Schwabe, 1995). The two-fingered protein family (ZPT2 family) is one of the largest subclasses of ZPT family. The zinc finger motif consists of 30 amino-acids sequences (CX2-4CX3FX5LX2HX3-5H) with 2 pairs of conserved Cys and His which is tetrahedrally coordinated to a zinc ion (Pabo *et al.,* 2001). Each zinc finger is a mini domain of approximately 30 amino acids that folds into a ββα motif in the presence of zinc (Figure 1.8). Tandem repeats of fingers are usually employed for DNA or RNA recognition, and DNA recognition is mediated by the α helix of each finger (Wolfe *et al.,* 2000). In case of plants, many of Cys-2/His-2-type zinc finger protein share similar structural features, where most fingers have a conserved six-amino acid stretch QALGGH which is essential for DNA binding (Takatsuji, 1999).

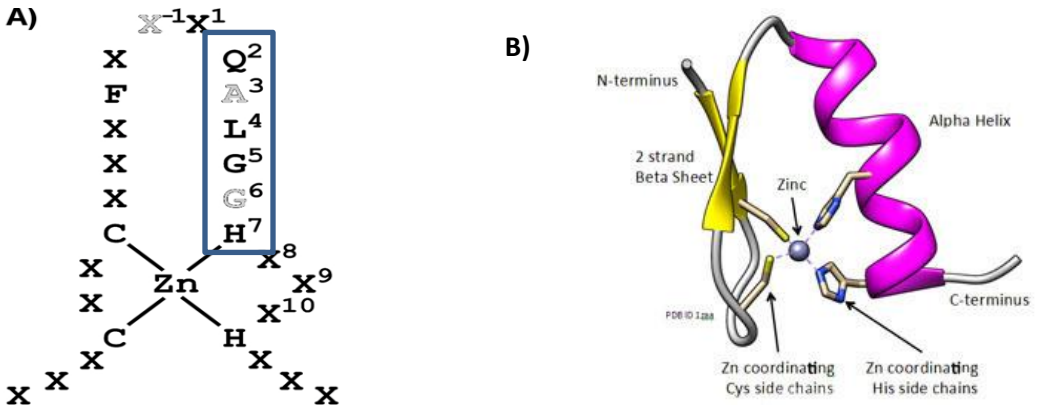


Figure 1.8: Structure of TFIIIA-type zinc finger proteins in plants. A. Zinc finger domain sequences. Positions of amino acids are numbered relative to the first putative helical residue and the conserved QALGGH sequence boxed. The three positions corresponding to the base-determinant positions in animal zinc-finger proteins are shown by white letters. B. Schematic representations of protein structure showing antiparallel Beta sheets (yellow), the alpha helix (pink) and the zinc molecule (blue) bound by two cysteine (Cys) amino acid and two histidine (His) amino acids (Protein Data Bank 2014).

1.7.2 C2H2 zinc finger transcription factors involved salt stress tolerance

There are 176 members in *Arabidopsis* and 189 members in rice which shows these are one of the largest families of transcription factors (Agarwal *et al.*, 2007; Ciftci-Yilmaz&Mittler, 2008) and several plant members are shown to have various regulatory roles in stress responses. There are some previous reports showing that some members of the gene family of Cys2His2-type zinc finger protein in *Arabidopsis*, *AtZAT10, AtAZF1*, *AtAZF2* and *AtAZF3* were upregulated by abiotic stress like drought, salt or ABA (Sakamoto *et al.*, 2000). (STZ/ZAT10) from Arabidopsis shows an increase in transcript level in response to NaCl exposure and overexpression of STZ suppressed plant development and improve resistance to heat, drought and salinity (Sakamoto *et al*., 2000). Another important report showed, in yeast calcineurin mutants which showed salt-sensitive phenotype when complemented with STZ, increased salt tolerance of transgenic yeast (Lippuner *et al*., 1996). There are various reports, example in wheat *(Triticum aestivum)*, TaZNF showed improved salt tolerance of transgenic *Arabidopsis* (Ma *et al*., 2016), *ThZF1* in salt stress (*Thellungilla halophila)* was induced by salinity and drought (Xu *et al.*, 2006), *GsZFP1* in soybean (*Glycine soja)*, were induced by

cold and drought (Luo *et al.,* 2012), and overexpression of *CgZFP1* from Chrysanthemum increased salt and drought tolerance in *Arabidopsis* (Gao *et al*., 2012). In rice, many studies have been done recently, several OsZFP genes were induced and showed improved tolerance to abiotic stress (Xu *et al*., 2008; Huang *et al.*, 2009; Zhang *et al.*, 2012, 2014). In rice, several members of the C2H2-type ZFPs, such as ZFP182, ZFP245, ZFP252 and ZFP179, have also been shown to be involved in the responses of rice to drought, salinity, and oxidative stress (Huang *et al*., 2007, 2009, 2012; Xu *et al.*, 2008; Sun *et al.*,2010).

In this study, we introduce two novel C2H2-type zinc finger transcription factors ZOS3-11 and ZOS3-12 which were isolated from rice cultivar Nihonmasari as we found highest homology with STZ in Arabidopsis and were highly induced under salt stress. They were identified to have two typical conserved QALGGH motifs and C-terminus of *ZOS3-11* and *ZOS3-12* gene contains typical Leu-rich L-box and DLN-box which play roles in protein interactions or in maintaining the folded structure. The DLN-box was thought to function in transcriptional repression.

1.8 Scope of study

Understanding the mechanism of high salinity stress and subsequently developing salinity tolerant new varieties with higher yield potential and stability across environments, climates and geographic locations can be a solution for increasing food production. In this study, we would like to have a small contribution with an innovative approach.

Considering the improved salt stress adaptation in the jasmonate mutants (*cpm2* and *hebiba*) and in plants overexpressing of JA metabolizing enzyme CYP94B3 leading to inactivation of jasmonate and also the fact that lack of jasmonate causes negative or detrimental effects on the plant development and defense owing to reduced fertility and increased susceptibility to pathogen and wounding response, we in our study want to have an innovative approach by suppressing the jasmonate response and regulating the temporal pattern of jasmonate signalling by dominant negative repression of jasmonate signalling with the aid of stable JAZ repressor specifically under high salinity thus avoiding the disadvantages of a general loss of JA signaling.

To address this topic the main objectives of my thesis were:

a) **Whether suppression of jasmonate signaling by overexpression of OsJAZ8 under the control of salt inducible promoter ZOS3-11 and ZOS3-12 leads to improved salt tolerance in rice and BY2 cell?**

   A study by Yamada *et al*., 2012, showed that transgenic rice plants overexpressing OsJAZ8ΔC (OsJAZ8 which lacks the Jas domain), exhibited a JA-insensitive phenotype. Taking this point into consideration, we here want to overexpress full-length and C-terminal (Jas domain) truncated JAZ8 protein under salt stress to alter salt tolerance. Expression of full-length and C-terminal (Jas domain) truncated JAZ8 protein was under the control of ZOS3-11 and ZOS3-12 promoter. The ZOS3-11 and ZOS3-12 promoter was selected based on the fact that, two C2H2 zinc finger protein in rice ZOS3-11 and ZOS3-12 showed close homology with STZ/ZAT10 and we found they are highly induced under salt and also regulated by jasmonates. As a proof of principle of our hypothesis, we investigated our concept by obtaining stable transgenic rice plants and stable transgenic tobacco BY-2 cells. Further their phenotypes were compared under salt stress. We could obtain some interesting outcomes when compared the BY2 transgenic cell lines treated with phytohormones like MeJA and IAA.

b) **Will characterization of C2H2 zinc finger transcription factor ZOS3-11 and ZOS3-12 help to uncover underlying gene-regulatory mechanisms?**

   In order to obtain more information regarding the two C2H2 zinc finger transcription factor ZOS3-11 and ZOS3-12 its localization and expression in rice was studied. In order to understand more on their link with jasmonate, ABA, and salt stress, gene expression studies were performed in wild type and JA biosynthesis mutants. DPI-ELISA assay was used to find its cis regulatory DNA sequence. Further we also show the transcription factors show repression activity using luciferase assay.

c) **Does comparison of salt uptake study and hormonal analysis in timely manner helps to understand mechanism of salt stress adaptation?**

   The understand more about salt uptake mechanisms, experiments were performed in natural conditions of greenhouse in IRRI (International Rice Research Institute). Na^+

and K^+ content was measured and compared in timely manner after salt treatment up to a week in Nihonmasari and jasmonate mutants *cpm2* and *hebiba*.

Further to unravel the fate of jasmonates, timely mannered hormonal analysis studies were performed. Hormones leading to biosynthesis and catabolism of jasmonates were analyzed when treated with salt.

2 MATERIALS AND METHODS

2.1 Preparation of plasmid overexpressing OsJAZ8 and OsJAZ8ΔC under the control of salt inducible promoters ZOS3-11 and ZOS3-12

2.1.1 Genomic DNA isolation

The genomic DNA of *Oryza sativa* L. ssp. Japonica cv. Nihonmasari was isolated to provide a template for the PCR amplifications of the promoter regions of gene *ZOS3-11* and *ZOS3-12*. The shoot from one-week old seedlings were cut into small pieces and immediately frozen in a 2 ml safe seal reaction vessel (Sarstedt, Nümbrecht, Germany) in liquid nitrogen. For DNA isolation, the frozen plant material was comminuted with a steel ball for 30 seconds at frequency of 22 hertz in the tissue lyser (Qiagen, Hilden, Germany). This was followed by the addition of 900 µl of boiling 1.5% CTAB buffer (chloroform-isopropanol) protocol described by (Sambrook&Russell, 2001). 900 µL preheated 1.5% CTAB buffer containing 150 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 30 mM EDTA, 2-Mercaptoethanol (8 µl/ml) (Roth, Karlsruhe, Germany) and polyvinylpyrrolidone (40 mg/ml) (Sigma, St. Louis, USA). Then the samples were incubated at 65 °C in a water bath for 1 hour with gentle vortex every 20 min. During this time, the CTAB buffer provides for the cell cleavage and the release of the nucleic acids. After that, 630 µl chloroform: isoamylalcohol (Roth) (24:1) was added before centrifugation. The samples were shaken at 1100 rpm for 15 minutes and then centrifuged at 13,300 rpm for ten minutes. Thereafter, 700 µl of the aqueous supernatant containing the nucleic acids were transferred to a 2 ml reaction vessel. For the nucleic acid precipitation 450 µl of ice-cold isopropanol (Roth) were added to the supernatant and inverted five times. In a further centrifugation step at 13,300 rpm for ten minutes, the nucleic acids were pelletized. The supernatant was discarded and the pellet was washed with 1 ml of 70% ethanol. After this washing step, the pellet was dried for 15 minutes at 30°C in the vacuum centrifuge. The DNA was then dissolved in 50 µl of 1/10 TE buffer (10x), to which 5 µg of RNase A (Qiagen, Hilden, Germany) was added for RNA digestion. After incubating the samples at room temperature for two hours, the respective concentrations were determined on the Nanodrop spectrophotometer (PEQLAB Biotechnology GmbH, Erlangen). For storage, the samples were frozen at -20 °C.

2.1.2 Cloning procedures

The promoter region of *ZOS3-11* (LOC_Os03g32220.1) and *ZOS3-12* (LOC_Os03g32230.1) and cDNA *OsJAZ8* (AK108738) and *OsJAZ8ΔC* (this C terminal truncated version was followed from Yamada *et al*., 2012) were cloned separately in the destination vector pMDC107 (Curtis *et al*., 2003) to obtain the final expression vector for stable transformation of tobacco BY-2 WT cells and rice. To clone the promoter region, Gateway cloning technology was used and for cloning *OsJAZ8* and *OsJAZ8ΔC*, T/A cloning method was used. The final T-DNA insert is shown in figure 2.1.

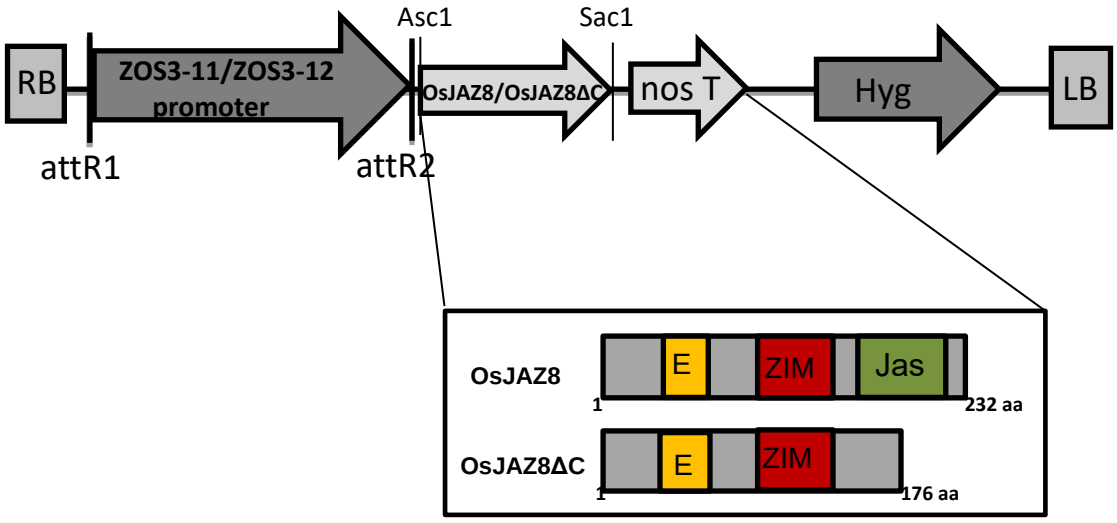


Figure 2.1: Schematic diagram of T-DNA inserts and OsJAZ8 and OsJAZ8ΔC domain constructs. T-DNA construct used for genetic transformation is shown, RB right border, LB left border, nos T- nos terminator, Hyg- Hygromycin resistance gene, attR1 and attR2 are gateway sequences flanking the promoter region, Asc1 and Sac1 restriction enzyme site flanks *OsJAZ8* and *OsJAZ8ΔC* region. OsJAZ8 protein (232 aa^{-}) have E domain, a ZIM domains (red) and Jas domains (green) and OsJAZ8ΔC (176 aa^{-}) lacks Jas domain in the C terminal

2.1.2.1 Cloning of OsJAZ8 and OsJAZ8ΔC

Second leaves of the 14 days old *Oryza sativa* L. ssp. japonica cv. Nipponbare were used to extract RNA using innuPREP Plant RNA Kit (Analytik Jena AG, Jena) and to synthesize cDNA which was performed with M-MULV Reverse Transcriptase (New England Biolabs,

Frankfurt am Main) using 1µg RNA as a template (Appendix 5.5). A full-length cDNA of *OsJAZ8* (AK108738) and Jas domain truncated (*OsJAZ8ΔC*) (according to Yamada *et al.*, 2012) was extracted by PCR amplification using primers with *Asc*I and *Sac*I restriction sites (Appendix 5.6.1). The PCR mix was used (Appendix 5.4.2 A) with 1 µl of a cDNA as template diluted fiftyfold and the PCR program (Appendix 5.4.2 B) with change in elongation time to 20 sec. A-tail was added to cleaned up PCR product incubating for 30 min in 72ºC with Taq DNA polymerase (5 U, New England Biolabs GmbH), ThermoPol® buffer and dATP (0.2mM) and ligated into the vector pGEM®T-Easy (Promega GmbH, Mannheim, Germany) vector using T4 DNA ligase (Promega GmbH) according to the manufacturers instruction. The positive product obtained was digested with *Asc* I and *Sac*I and then ligated into the *Asc* I and *Sac*I sites of the destination vector pMDC107 (Curtis *et al.*, 2003) replacing GFP coming in the downstream position of the promoter to create recombinant plasmid pMDC107-OsJAZ8/OsJAZ8ΔC for expressing the fusion protein. Positive plasmids were confirmed by restriction analysis, and further verified by sequencing (primers used shown in Appendix 5.6.2)

2.1.2.2 Cloning of promoter region of ZOS3-11 and ZOS3-12

Promoter regions were inserted into the destination vector pMDC107-OsJAZ8/OsJAZ8ΔC using the Gateway®-Cloning technology (Invitrogen Corporation, Paisley, UK). The promoter region (2000-3000 bp upstream of the start codon) of *ZOS3-11* (LOC_Os03g32220.1) and *ZOS3-12* (LOC_Os03g32230.1), were extracted and amplified from the isolated genomic DNA using specific primers designed by Primer 3plus online software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi, last accessed 21/09/17). In addition, the attB sequences for the Gateway® cloning have been added to these primers. The PCR primers designed with the Gateway® attB sequences are listed in Appendix 5.6.1. For PCR, the enzyme Q5® High-Fidelity DNA polymerase from New England Biolabs (Frankfurt am Main) was used. In contrast to the Taq DNA polymerase, this polymerase has a 3'-5 'exonuclease activity and thus has a significantly lower error rate. The individual components of the 50 µl reaction mixture are listed in Appendic 5.4.1 A. The PCR program was adapted to the Q5® High-Fidelity DNA polymerase and is shown in Appendic 5.4.1 B.

The NEB - TM Calculator v. 1.9.4 was used to calculate the appropriate annealing temperature for the respective primer pair.

The PCR products prepared for the promoter regions were admixed with 5x loading buffer and the complete reaction mixture was then loaded onto a 1% agarose gel. The 1 kb DNA ladder from New England Biolabs (Frankfurt am Main) served as a size marker. After running the gels at 100 volts for 20 min, the PCR fragments with the correct size were excised from the gel using a scalpel. Subsequently, the PCR products were purified using the kit NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Düren, Deutschland) according to the manufacturer's instructions. The samples were eluted with nuclease-free water and the concentrations were measured at the Nanodrop spectrophotometer.

In Gateway®-Cloning, the BP reaction was used according to the manufacturers instruction to recombine the amplified attB-flanked promoter region with the attP-equipped donor vector pDONR ™ /Zeo (Thermo Fisher Scientific Inc., Waltham, MA, USA). Mini prep was performed using Roti®-Prep Plasmid MINI (Carl Roth GmbH + Co. KG, Karlsruhe) with the positive colonies to get the plasmid. Positive plasmids were confirmed by restriction analysis and further verified by sequencing (GATC Biotech, Cologne, Germany) before proceeding for the LR reaction. Further LR reactions were performed to transfer insert from entry clones to destination vector pMDC107-OsJAZ8/OsJAZ8ΔC. LR clone selection was the same as BP clone selection except for the substitution of Ampicillin (100 μg/ml) for Zeocin (50 μg/ml) in the LB plates.

2.2 Establishment of stable transgenic tobacco BY-2 cells, growth conditions and experiments performed

Tobacco BY-2 (*Nicotiana tabacum* L. cv BY-2) suspension cultures ( ) were used for this study.

2.2.1 Agrobacterium-mediated transformation

Different transgenic stable cell lines ZOS3-11::JAZ8, ZOS3-11::JAZ8ΔC, ZOS3-12::JAZ8 and ZOS3-12::JAZ8ΔC were obtained through method developed by Buschmann *et al*. (2010) with several modifications for better performance. 1.5 ml of 7-day-old BY-2 WT cells was

used during sub-cultivation and kept for 3 days under the same conditions as normal suspension cell culture. Afterwards, 3 flasks each containing 1.5 ml cell culture were pooled together and washed thrice with 200 ml of Paul's media (4.3 g/l Murashige and Skoog salts (Duchefa Biochemie, Haarlem, the Netherlands), 10 g/l sucrose, pH 5.8) each time. The washing steps were performed using a scientific Nalgene® filter holder (Thermo Scientific, Langenselbold, Germany) combined with Nylon mesh with pores of diameter of 70 µm. The washed cells were then resuspended in 15-18 ml of Paul's medium yielding a 5-to 6-fold concentrated cell suspension. 6 ml of these concentrated cell suspension was mixed with cell suspension of transformed *Agrobacterium tumefaciens* prepared as following.

100 µl chemo-competent *A.tumefaciens* (strain LBA4404; Invitrogen Corporation, Paisley, UK) was thawed on ice and incubated with 100 ng expression vectors containing genes of interests on ice for another 20 min. After 37 °C heat shock transformation and 28°C for 90 s and incubation for 1.5~2h, bacteria were spread onto solid LB (Lennox Broth, Roth, Karlsruhe, Germany) medium containing corresponding antibiotics (50 µg/ml rifampicin, 300 µg/ml streptomycin, and 100 µg/ml kanamycin), and incubated for 3 days at 28°C in the dark. Afterwards, one of the single colonies was inoculated into 5 ml LB liquid medium supplied with the same selective antibiotics and incubated at 28 °C agitated vigorously overnight. The OD600 of the overnight culture was determined and 1 ml of the overnight culture was inoculated into 5 ml of fresh LB-medium (without antibiotics) to reach an OD600 of 0.15. After approximately 5 h of growth, 6 ml of the transformed *A. tumefaciens* bacteria were harvested at an OD600 of 0.8 by centrifugation at 8000 g (HeraeusPico 17 Centrifuge, 600 Thermo Scientific, Langenselbold, Germany) for 7 min in a 50 ml falcon tube at 28°C. The bacteria were then resuspended in 180 µl washing medium (Paul's medium) by mixing vigorously using a bench-top vortexer (Bender & Hobein Zurich, Switzerland) till the suspension becomes homogeneous.

Mixture of BY-2 cells with the homogenized bacteria suspension was prepared and incubated in the falcon tube on an orbital shaker at a 30°angle above the horizontal at 100 rpm for 5 min till fully mixed. Subsequently, this mixture was dropped with sterile oblique cut tips onto petri dishes containing washing medium (Paul's media) solidified with 0.5 % (w/v) Phytagel (Sigma P8169) without any antibiotics on which a single layer of sterile filter paper was placed in advance. Those plates were sealed with parafilm and incubated at 22 °C in the dark instead of

28°C as recommended in the original publication. Cells were subsequently transferred on MS agar plates (MS media with 0.8% Danish agar) containing 300 m/l cefotaxime and 40 mg/l hygromycin. After 14-21 days, resistant calli were pooled to obtain sufficient material for starting a suspension culture. The presence of inserts was confirmed using PCR (Appendix 5.6.2). Genomic DNA was extracted from BY2 suspension cells using CTAB method (as explained in section.2.1.1) The concentration and quality was checked using a nanodrop spectrophotometer.

2.2.2 Cell cultures

The positive calli were transferred to 30 ml MS liquid media containing 4.3 g/l Murashige and Skoog salts (Duchefa Biochemie, Haarlem, the Netherlands), 30 g/l sucrose, 200 mg/l KH_2PO_4, 100 mg/l inositol, 1 mg/l thiamine, and 0.2 mg/l 2,4-D, pH 5.8. The cells were sub-cultivated on a weekly basis by inoculating 1.0 ml of stationary cells into 30 ml fresh medium contained in 100 ml Erlenmeyer flasks using oblique cut tips. The cells were incubated at 26 °C in darkness on an orbital shaker (IKA Labortechnik, Staufen, Germany) rotating constantly at 150 rpm. The stock BY-2 calli were maintained on the same medium solidified with 0.8 % (w/v) agar (Roth, Karlsruhe, Germany) and sub-cultivated monthly. Transgenic suspension cells and calli were cultivated on the same medium as mentioned above with Hygromycin 40mg/ml.

2.2.3 Phenotyping and experimental methods used

To test for potential differences in the response of the transgenic line, different stress treatments were employed. As abiotic stress, salinity was used, administering 50, 100, or 150 mM of NaCl at the time of sub-cultivation. To check the effect of jasmonate, the cells were also treated with 100 µM MeJA at the time of sub-cultivation.

2.2.3.1 Determination of cell mortality, packed cell volume, cell length and cell cycle

To quantify cell mortality, aliquots of cells from each cell lines were collected after 24 h treatment and the medium was drained in a custom-made staining chamber using a mesh with a pore-size of 70 µm as bottom (Nick *et al.*, 2000), and then transferred into 1 ml of 2.5 % Evans Blue (w/v) (Sigma-Aldrich) dissolved in deionised water for 1 min. After washing 3

times with fresh distilled water the cells were viewed under AxioImager Z.1 microscope (Zeiss, Jena, Germany) and mosaic pictures were taken. Cell mortality was calculated as the number of dead cells divided by the total number of cells. For each experiment 1000 cells were counted for each cell line. The percentage of dead cells were calculated and plotted. Each data point represents mean and standard error obtained from at least three independent experimental series. The results were tested for significance using Student's *t*-test at 95 % and 99 % confidence levels.

Growth of the BY-2 cell culture was approximated by measuring the packed cell volume (PCV) (Jovanovic *et al.,* 2010) at day 4 after sub-cultivation/treatment. 14 ml of cell culture were poured from the Erlenmeyer flasks directly into a 15 ml falcon tube and kept vertically at 4 °C for a time period between 48 h and 72 h (depending on the density of the cell culture) till the upper surface can be clearly distinguished from the cells sediment and the PCV were read directly using the scale of the 15 ml falcon. The percentage of the packed cell volume were calculated from the measurement and graphs were plotted. Each data point represents mean and standard error obtained from at least three independent experimental series. The results were tested for significance using Student's *t*-test at 95 % and 99 % confidence levels.

To find the effect of treatment on cell length, 500 µl aliquot of the cells were collected after 4 days and 7 days after the treatment viewed under AxioImager Z.1 microscope (Zeiss, Jena, Germany). For each picture, the MosaiX module of the AxioVision software was used to cover a 4x4 mm area with 121 single pictures at an overlay of 10 % to monitor division synchrony (Campanoni *et al.*, 2003; Maisch&Nick, 2007). Cell length was measured from the image of the central section of the cells using the length measurement function of the AxioVision software according to (Maisch&Nick, 2007). And each data point represents mean and standard error from at least 500 individual cells obtained from three independent experimental series. The results were tested for significance using Student's *t*-test at 95 % and 99% confidence levels.

The average length of the cell cycle was estimated from the time course of cell density estimated by a hematocytometer (Fuchs-Rosenthal), using an exponential model for proliferation (Nt = N0.e kt with Nt cell density at time point t, N0 cell density at inoculation, and k the time constant). In order to set the reference, the starting number (N0) was quantified just after sub-cultivation.

2.2.3.2 Effect of auxin on cell length

The cells were treated with auxin, indole-3-acetic acid (IAA; Roth, Karlsruhe, Germany) (0.3,1, 3 and 10 µM) on the 4th day after subculture (when cells reach stationary phase). Cells treated with sterilized water was used in as control. For cell length, 500 µl aliquot of cells from each sample were collected on the 4th day before and the 7th day after auxin treatment and viewed under AxioImager Z.1 microscope (Zeiss, Jena, Germany) to obtain the images. Cell length were measured as mentioned in section 2.3.3.1. Each data point represents the relative increase in the cell length from 4th (before IAA treatment) to 7th day from at least 500 individual cells obtained from three independent experimental series.

2.3 Establishment of stable transgenic rice, growth conditions and experiments performed

The *japonica* variety ***Kitake*** of rice (*Oryza sativa* L.) was used for this study. The transgenic lines were generated at the Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement (CIRAD, Montpellier, France) according to the method mentioned in (Sallaud *et al.,* 2003).

T2 generation seeds of transgenic plants (ZOS3-11::JAZ8, ZOS3-11::JAZ8ΔC, ZOS3-12::JAZ8ΔC) were used for stress treatments. The lines used for experiments are shown in (Appendix 5.9) After 10 days of germination in phytoagar, the seedlings were transferred to hydroponic condition and placed in a growth chamber continues light condition at 25℃ and 75% relative humidity. After two days, the seedlings were used for the following salt stress treatments. For phenotyping, seedlings were treated with 100 mM NaCl solution. Two days later, length of whole shoot, 2nd leaf and 3rd leaf were measured and photographed. And also, percentage increment of the length was calculated. All above experiments were repeated three times.

2.4 Investigation of *OsJAZ8* and *OsJAZ8ΔC* expression levels in the transgenic BY-2 and rice

To study the expression of *OsJAZ8* and *OsJAZ8ΔC* in transgenic BY-2 cells, 2 ml of the treated BY-2 cells were collected at a time interval of 1h, 6 h and 24 h after treated with 150 mM salt, 100 µM MeJA and control (water). The liquid media from the cells were removed using filter paper and were transferred into a 2ml eppendorf tube and immediately frozen in liquid nitrogen for further process. 5 mm steel bead was used to lyse the cells in a TissueLyser (Qiagen). All cDNA samples were analyzed in triplicate from three sets of independent biological replicates. The data were expressed as mean ± standard error.

To study the expression of *OsJAZ8* and *OsJAZ8ΔC* in transgenic rice, leaf samples from at least three plants for one sample were collected at a time interval of 6 h and 24 h after treated with 100 mM of NaCl and control (no treatment). For expression analysis of *OsJAZ8* and *OsJAZ8ΔC* genes (Appendix 5.6.3), total RNA extraction, reverse transcription into cDNA and real-time quantitative PCR was performed is explained in section 2.5 and 2.6. All cDNA samples were analyzed in triplicate from three to five sets of independent plants. The data were expressed as mean ± standard error.

2.5 RNA extraction and cDNA synthesis

RNA extraction was performed using the InnuPrep plant RNA kit (Analytik Jena RNA kit) according to the manufacturer's instructions. 5 mm steel bead was used to lyse the cells in a TissueLyser (Qiagen). On-column digestion of genomic DNA was performed with RNase free DNAse I (Sigma) according to the manufacturer instruction. Purity and integrity of RNA were checked by agarose gel electrophoresis. The cDNA synthesis was performed with M-MULV Reverse Transcriptase (New England Biolabs, Frankfurt am Main) using 1µg RNA as a template (Appendix 5.5).

2.6 Real-time PCR

Real time (qPCR) was performed with the CFX96 Touch ™ Real-Time PCR Detection System from Bio-Rad Laboratories GmbH (Munich) in 96-well plate for a reaction volume of 20 µl with the program as follows: 95 ◦C for 3 min, and 39 cycles (95 ◦C for 15 s, annealing at 60 ◦C for 40 s and extension at 72 ◦C for 30 s). The chemicals used the reaction mixture qPCR analysis contained 200 nM of each primer, 200 µM of each dNTP, 1X GoTaq colorless buffer, 2.5 mM $MgCl_2$, 0.5 U GoTaq polymerase (Promega, Mannheim, Germany), 1x SYBR Green I (Invitrogen, Darmstadt, Germany), and 1 µl of a cDNA template diluted tenfold. The oligonucleotide primer sequences for the housekeeping and genes of interest are listed in Appendx 5.6.3. To compare the transcript levels between different samples, the Delta-delta Ct method was used (Livak and Schmittgen, 2001). GADPH was used as endogenous control for normalization in case of BY2 cells and Ubiquitin 5 and Ubiquitin 10 in case of rice. At least three biological replications were performed for each treatment.

2.7 Characterization of C2H2 transcription factor ZOS3-11 and ZOS3-12

2.7.1 Localization of ZOS3-11 and ZOS3-12

For finding the localization of ZOS3-11 (LOC_Os03g32220.1) and ZOS3-12 (LOC_Os03g32230.1), these were expressed in fusion with GFP by biolistic transformation in rice coleoptiles (cv. Nipponbare) and BY-2 cell. The vector constructs having GFP were made according to the manufacturer's instruction in the Gateway Cloning Technology booklet (Invitrogen). The coding sequences of *ZOS3-11* and *ZOS3-12* were extracted and amplified using specific primers designed by Primer 3 online software. Gateway attB sequences were added at the 5' end of both forward and reverse primers (FP-5-′GGGGACAAGTTTGTACAAAAAA GCAGGCTATGGTGACCAACATGACCCA-3′ and RP:5′GGGGACCACTTTGTACAAG AAAGCTGGGTCTAAATTAAGCTCCAATGATGGGC -3′ for *ZOS3-11* and FP: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGATATGACGGCCGCCCTG -3′ and RP:5′- GGGGACCACTTTGTACAAGAAAGCTGGGTCGTTGGACATCCTGAGCTT CTTG-3′ for *ZOS3-12*). Further LR reactions were performed to transfer insert from entry

clones to destination vector p2GWF7 (Karimi *et al.*, 2002). LR clone selection was the same as BP clone selection except for the substitution of Ampicillin (100 µg/ml) for Zeocin (50 µg/ml) in the LB plates. Plasmids p2GWF7ZOS3-11 und p2GWF7ZOS3-12 were used to perform the biolistic transformation. For biolistic transformation, the rice seeds were grown on floating plastic nets for four days in the dark as described by (Holweg *et al.*, 2004). The seedlings were adhered on the edge of a glass slides with the adhesive Secure Silicone Adhesive B-400 (Factor IIInc., Lakeside, AZ, USA). The rest of the procedure is explained in (Section 2.8).

2.7.2 Protein expression, extraction and DPI-ELISA assay

2.7.2.1 Protein extraction

For protein-DNA interactions screening, the protein was expressed in *E. coli* strain BL21/RIL (DE3). The His-tag fusion constructs for the protein expression in bacteria were built via gateway cloning as mentioned before. *ZOS3-11* and *ZOS3-12* were cloned into the binary plasmid pET-DEST42 containing C-terminal His-epitope. The construct was transformed in *E. coli* strain BL21/RIL (DE3) by heat shock method and the positive clones were selected by Ampicillin (100 µg/ml). As negative control, we used BL21 cells transformed with *pET-DEST42-empty* vector without *ccDB* cassette. The proteins were expressed under the control of the bacteriophage T7 promoter. The positively transformed colony was incubated in 5 ml culture flasks containing LB medium supplemented with ampicillin overnight. Before the induction, the pre-cultures were transferred to 500 ml LB media with start OD of 0.1 and grown to an OD 600 of 0.6-0.8 at 37 °C and shaken at the speed of 180 rpm. After that the expression was induced by the addition of 500 µM of IPTG. The cells were collected by centrifugation (2500 g, 20 min, 4°C) (Hermle Universal centrifuge, Wehingen, Germany) at an optical density $E_{600} = 1$ (2-4 hours) and washed (10 mM Tris-HCl pH7.5-8, 100 mM NaCl). The cell sediment was resuspended in protein extraction buffer (4 mM HEPES pH 7.5, 100 mM KCl, 8% (v/v) glycerol, 1 mM PMSF (stock 100 mM) and proteinase inhibitor cocktail (Roche, Germany)). Recombinant protein extraction was performed by sonication (UP100H, Hielscher, Germany) on ice with the frequency of 6 cycles of 15 s with 15 s interruption, 80% power; and centrifuged at 4000 g at 4 °C for 20 min to separate supernatant (crude extract) and pellet. The total protein

amount of the supernatant (crude extract) was measured *via* Bradford Assay (BioRad). To check the soluble and unsoluble part of the crude extract, an aliquot was centrifuged for 15 min for 10000 rpm. The coresponding pellet was used as unsoluble part. 5 vol of ice cold acetone was added to supernatant and kept for 30 min at -20°C. Then it was centrifuged at 10000 rpm for 15 min. The corresponding pellet represents the soluble part. The protein extracts were kept either at 4°C ready to use or at -20°C. If stored at -80°C, glycerol is added to a final concentration of 20% (v/v). The soluble and unsoluble parts were verified by SDS-PAGE and western blotting.

2.7.2.2 Electrophoresis with SDS-Polyacrylamide gel

The components for discontinuous SDS-polyacrylamide gels used for protein separation are shown in Appendix 5.7 (Laemmli, 1970). The soluble and unsoluble samples were mixed with 3x sample buffer (30% (v/v) glycerine, 300 mM DTT, 6% (w/v) SDS, 48% (v/v) stacking gel buffer, 0.01% (w/v) bromophenol blue). They were incubated at 95 °C for 5 min. The proteins were loaded and were separated equally on two SDS-polyacrylamide gels, in a mini PAGE chamber (Atto, Tokyo, Japan) containing running buffer (25 mM Tris, 192 mM glycine, 0.15% (w/v) SDS) at 25 mA supplied by an electrophoresis power supply PHERO-stab 300 (Biotec-Fischer, Germany) per gel for 90 min. Color Prestained Protein standard (Broad Range 11-245 kDa, New England Biolabs, Frankfurt, Germany) were used as protein ladder. One of the gels was stained in Coomassie staining solution (0.04% (v/v) Coomassie Brilliant Blue R250, 40% (v/v) methanol, 10% (v/v) acidic acid) for 1 hours and destained in 30% (v/v) ethanol supplemented with 10% (v/v) acetic acid for another 1 hours. Then gels were scanned using a HP ScanJet 3400C (Hewlett-Packard, Palo Alto, USA) for the documentation, and they were dried for long term storage. The other gel was used for western blotting to confirm the presence of required protein.

2.7.2.3 Western blot

Polyvinylidene fluoride (PVDF) membrane (Pall Gelman Laboratory, Dreieich, Germany) and 4 pieces of blotting papers (Whatman, Dassel, Germany) cut in the size of gel were used.

Polyvinylidene fluoride (PVDF) membrane, which was activated by incubation in methanol (Roth) for 1 min and blotting paper was soaked for 5 min in transfer buffer (14.4 g/l glycine, 12.07 g/l Tris-HCl, 20% (v/v) MeOH) before starting the blotting process. Proteins were transferred onto the membrane with Trans-Blot® Semi-Dry Transfer Cell (Bio-Rad) a constant current of voltage 20 V for 60 minutes per gel. After blotting, the membrane was blocked for 60 min, with 4% (w/v) blocking buffer ie, 4% milk in TBS buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl). The transferred protein on membrane was rinsed for 10 min twice in TBST buffer (TBS buffer, Tween-20) and one time in TBS buffer. The blot was then incubated overnight at 4 °C with primary antibody as a monoclonal mouse anti-histidine antibody 1:2000 (Penta His Antibody, BSA-free, Qiagen, 1:2000 diluted in TBS buffer). After washing, the membrane was incubated with the secondary antibody (Anti-mouse IgG, alkaline phosphatase-conjugated (Sigma, St. Louis, USA), 1:50000 diluted in TBS buffer) for 60 min. The secondary antibody was washed away by rinsing for 10 min in TBST for 4 times. The signal was developed with an alkaline phosphatase-based development method. The membrane was incubated in staining buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.7) freshly supplemented with 1/10 magnesium stock (500 mM) for 15 min prior to the development. The membrane was developed in 5 mL developer solution consisting of 66 µL nitrobluetetrazolium (NBT; 75 mg/ml in 75% [v/v] dimethylformamide, Thermo scientific) and 33 µl 5-bromo-4-chloro-3-indoxylphosphate-p-tuloidin (BCIP; 50 mg/ml in 75% [v/v] dimethylformamide (Thermo scientific) which were diluted freshly with 5 ml staining buffer with 1/10 magnesium stock solution for 1 min. The reaction was stopped by rinsing in water. The blots were dried and scanned for documentation.

2.7.2.4 DPI-ELISA assay

DPI-ELISA assay was used to elucidate the cis-acting DNA binding sequence of the two C2H2 zinc finger transcription factor ZOS3-11 and ZOS3-12 which was performed in University of Tübingen. The crude extraction from *E. coli* (Strain: BL21(DE3)RIL) cell culture including protein pDEST42-ZOS3-11 and pDEST42-ZOS3-12 were used. The workflow of the DPI-ELISA was modified from Brand et al. (2010) (Figure 2.2). DPI-ELISA screen were prepared by binding 10 µl ds-bio DNA probes streptavidin-coated plates by incubating with 20 µl TBST

buffer at 37°C for 1 hour. After washing with TBST for 3 times, micro-well plate was blocked with 5% non-fat dry milk (Roth, Germany). 125 µg crude protein extract was added for the binding of immobilised ds-bio DNA in each well. After washing, 30 µL α-His-HRP antibody (Qiagen, Hilden, Germany) diluted 1:1000 in PBS-T was added for incubation at 22 °C for 1 hour. Photometric detection (peroxidase reaction) was carried out via ELISA-reader in less than 1 hour. The relative unit data was calculated by normalization of the mean of two independent samples and standard deviation to the negative control.

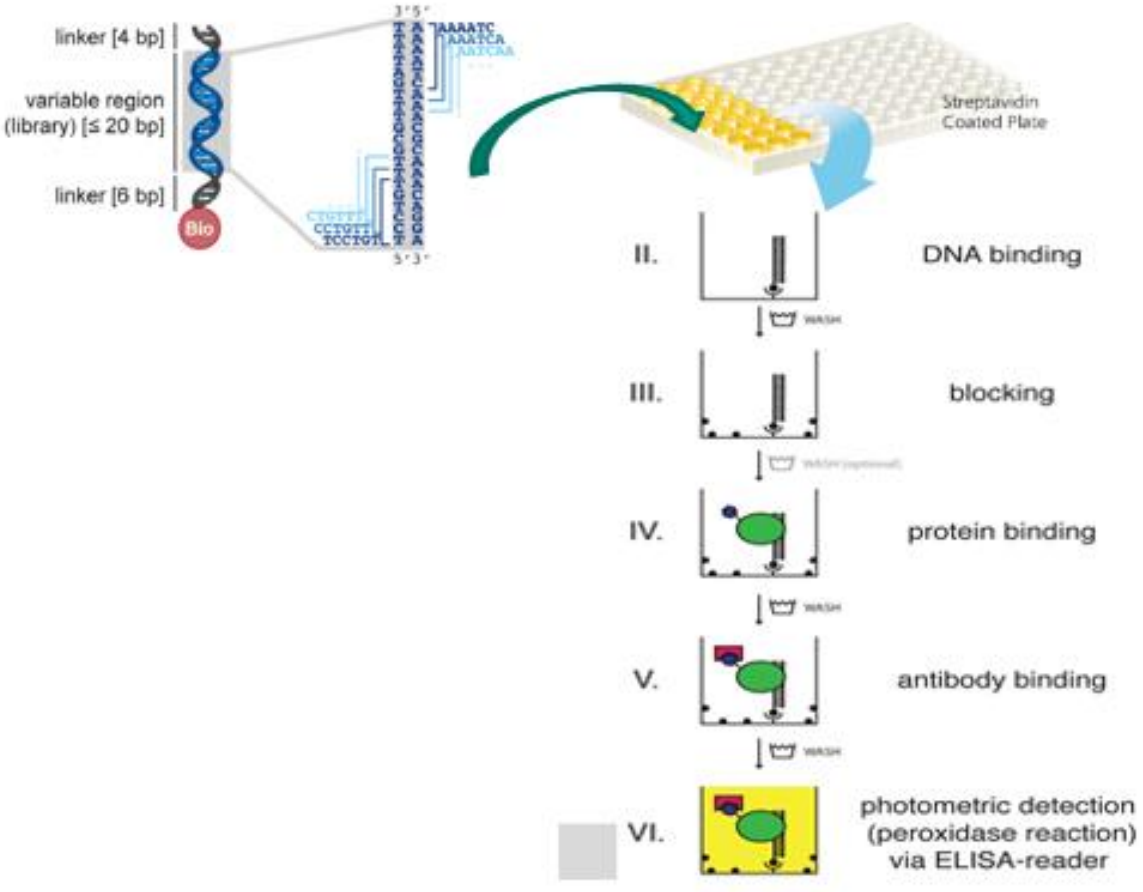


Figure 2.2: Schematic explanation of the DPI-ELISA library screening system. Image source: (Brand *et al.*, 2013)

2.7.3 Comparison of *ZOS3-11* and *ZOS3-12* expression by NaCl and ABA

In this study *Oryza sativa* L. ssp. japonica cv. Nihonmasari was used as the wild type and its jasmonate mutant lines *cpm2* generated in the same cultivar (Riemann *et al*. 2013) were used.

The caryopsis was dehusked and surface sterilized by incubating the seeds in 70% ethanol for 1 min then washed briefly 3 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 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 floating racks and moved to a glass container containing ultrapure water for one day. To compare the expression of *ZOS3-11* and *ZOS3-12* in WT and *cpm2*, 150mM of salt and (0.1, 1 and 10 µM) of ABA was treated to the 12 days old plants. Leaf and root samples were collected 1h, 6h and 24h after the salt treatment and root samples in case of ABA treatment. RNA extraction from the leaf material was carried out using the innuPREP Plant RNA kit (Analytik Jena AG, Jena) and the RNA from the root material was extracted with the Spectrum ™ Plant Total RNA Kit (Sigma-Aldrich Chemie GmbH, Munich) (Section 2.5). Gene expression in leaves and roots were compared using qRT-PCR (section 2.6). EF-1α and UBQ5 were used as endogenous control for normalization in case of NaCl and UBQ5 in case of ABA. At least three biological replicates were performed for each treatment.

2.8 Assay of promoter activity by dual-luciferase assay system

Activity of *ZOS3-11*, *ZOS3-12*, *OsJAZ11* and *OsJAZ12* promoter were checked in this experiment. Plasmids were created having the promoters fused with Luciferase gene (LUC) (Appendix 5.3). A well-established dual-luciferase system based on transient transformation (Duan *et al.,* 2016) was employed to measure promoter activation. The *Renilla* luciferase plasmid pRluc was transformed as an internal control in parallel to calibrate the firefly luciferase luminescence against variations of transformation (Horstmann *et al.,* 2004). The experiment was performed in two main steps 1) transient transformation using biolistic bombardment and 2) dual-luciferase assay.

2.8.1 Biolistic transformation

A transient transformation system was performed using biolistic bombardment: gold particles (1.5 –3.0 µM; Sigma-Aldrich, St. Louis, USA) were coated with plasmid DNA, including 50 ng of specific promoter DNA and 100 ng control plasmid pRluc, according to the standard manual of Bio-Rad (PDS-1000/He Particle Delivery System manual). DNA-coated particles were then loaded onto macrocarriers (Bio-Rad Hercules, CA USA) and transferred into a custom-made chamber for shooting cells.

Three-day-old cells from WT BY-2 cells placed on solid MS medium (0.8 % Danish agar) were used to check the activity of *ZOS3-11* and *ZOS3-12* promoters. And young leaves from one month old rice plant (Japonica variety Paw san yin) were used to check the activity of *OsJAZ11* and *OsJAZ12* promoters. The cut leaves were placed on petri dishes with 0.4% phytoagar. Four leaf blades were placed in the center of the bowl with the leaf bottom upwards. The plant materials were then transiently transformed through three shots at a pressure of 1.5 bar in a vacuum chamber of -0.8 bar, as described in (Maisch *et al.*,2009).

2.8.2 Luciferase assay

In case of BY-2 cells, promoter *ZOS3-11* and *ZOS3-12* activation was measured in response to elicitation by either 100 mM NaCl or 100 µM MeJA, or a related solvent without elicitor (as control) administered 24 h after bombardment. Cells were harvested 24 h after treatment and homogenised in 150 µl of 2 × passive lysis buffers (PLB, Promega, Madison, WI) by grinding on ice with a pestle and mortar for 1.5 min.

In case of rice leaves, promoter (*OsJAZ11* and *OsJAZ12*) activation was measured in response to elicitation by 100 µM MeJA. The treatment was done 24 h after bombardment. The next day, the leaf blades were harvested in SafeSeal reaction vessels (Sarstedt, Nübrecht, Germany), frozen in liquid nitrogen, and pulverized by means of a steel ball in the tissue lyser for 30 seconds at 22 hertz and was then added with 150 µl of 2 × passive lysis buffers (PLB, Promega, Madison, WI).

After centrifugation of the lysates for 2 min at 10,000 rpm, measurement of the luciferase activitiy was performed with the dual-luciferase reporter assay system (PJK, Kleinbittersdorf,

Germany) by sequential addition of 50 µl Beetle juice and Renilla Glow Juice to individual 20 µl samples of the lysate supernatant. Luminescence was measured using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad Germany). All experiments were performed in triplicate and all experiments were repeated in three independent series. Mean values of the ratios between firefly and *Renilla* luciferase luminescence were recorded as a readout of luciferase activity, normalised for transformation efficiency, and relative changes of activity calculated over the values measured in the solvent-treated samples. pLuc Vector containing the promoter of actin was used as positive control.

2.9 Comparison of salt uptake in jasmonate mutants and its WT

2.9.1 Plant materials, growth and stress conditions

These experiments were performed in glasshouse at the International Rice Research Institute (IRRI), Los Baños, Philippines (Los Banõs, Laguna, 14°10'11.81"N, 121°15'39.22"E). In this study *Oryza sativa* L. ssp. japonica cv. Nihonmasari was used as the wild type, two mutant lines *cpm2* and *hebiba* used were generated in the same cultivar (Riemann *et al.,* 2013), seedling-stage tolerant and sensitive varieties FL478 and RC222 (obtained from IRRI) were used. After breaking the dormancy, the seeds were germinated without dehusking by keeping it on petridish with moistened filter papers for 3-5 days in 35ºC. The pre-germinated WT, FL478, RC222 and selected *cpm2* and *hebiba* seeds were sown (one seed per hole) on a styrofoam sheet having 100 holes with a nylon net bottom in the two sets, one for the control and the other for salinity stress (as shown in figure 2.3). The experimental layout was with three replications for salinity stress and three replications for non-saline control. The sheets were left to float in a container containing Yoshida solution (Yoshida *et al.,* 1976), with slight modifications as per the protocol by (Singh&Flowers, 2011) before addition of salt. After 10 days 100mM of NaCl was added to obtain electrical conductivity EC, of 10 dSm-1. Samples were collected for 7 days after treatment. The pH of the nutrient solution was maintained at approximately 5 and the nutrient solution was renewed weekly throughout the experiment. The seedlings were grown in the phytotron facilities at IRRI that are maintained at 29/ 21°C day/night temperature and at a minimum relative humidity of 70%. Irradiance inside the glasshouse was maintained at least 80% of incident solar radiation

Figure 2.3: Experimental setup for salt treatment in IRRI

2.9.2 Na^+ and K^+ content determination

To investigate the Na^+ ion accumulation in shoots,10 days rice plants (salt tolerant variety: FL478, WT: Nihonmassari, Jasmonate mutants: *cpm2*, *hebiba*, salt sensitive variety: RC222) were exposed to a nutrient solution containing 100 mM NaCl for 7 d as explained above. The leaf blades 4 to 6 (from old to young) were separately sampled for ion analysis. The leaves were incubated in 20 ml 100mM glacial acetic acid at 90ºC for two hours in a water bath. The leaves were dried to get the dry weight. Na^+ concentration was measured in an Atomic Absorption Spectrometer (Perkin Elmer Analyst 200). Estimation of Na^+ in µmol/mg dry weight was using the equation:

$$Na+ (\mu mol/mg\ DW) = [C * V/23]/DW$$

Where, C is flame photometer reading, V is extraction volume (ml) and DW is oven dry weight (mg).

2.10 Hormonal analysis

Oryza sativa L. ssp. japonica cv Nipponbare were treated 100 mM salt. The rice plants grew for 7 days in sterile phytoagar and 5% MS, were then cultivated in glass containers with racks and after a 3-day rest period, salt stress in the form of 100 mM NaCl. In parallel, the control plants continued to grow in pure 5% MS. Sampling was done with 2nd leaf of the plants, took place at the times 6 h, 24 h and 72 h after stress treatment. To determine the content and jasmonic acid and jasmonic acid conjugates as well as possible degradation products of salt stress, hormone extraction on leaf samples were done and these then analyzed by UPLC-MS/MS by Dr. Thierry Heitz (IBMP, Strasbourg, France). Hormone extracation was performed according to (Widemann *et al.*, 2013), hormone anaylsis like described in (Smirnova *et al.*, 2017).

3 RESULTS

In this chapter, the results will be presented in three main sections. The first part deals with the characterization of the ZOS3-11 and ZOS3-12 transcription factor, namely localization, the effect of salt and ABA on its expression, finding cis-regulatory elements and confirmation of its binding and repression activity. Following that in the second part, the result focuses on the main part of my work, firstly, examining different phenotypic parameters in response to salt stress, methyl jasmonate (MeJA) and auxin on the transgenic BY-2 cells. Secondly, phenotypical studies in transgenic rice under salt stress. The third part deals with some extra investigations about hormonal analysis and sodium uptake studies in rice and jasmonate mutant under salt stress as an extension to previously reported study.

3.1 Characterization of ZOS3-11 and ZOS3-12 transcription factor

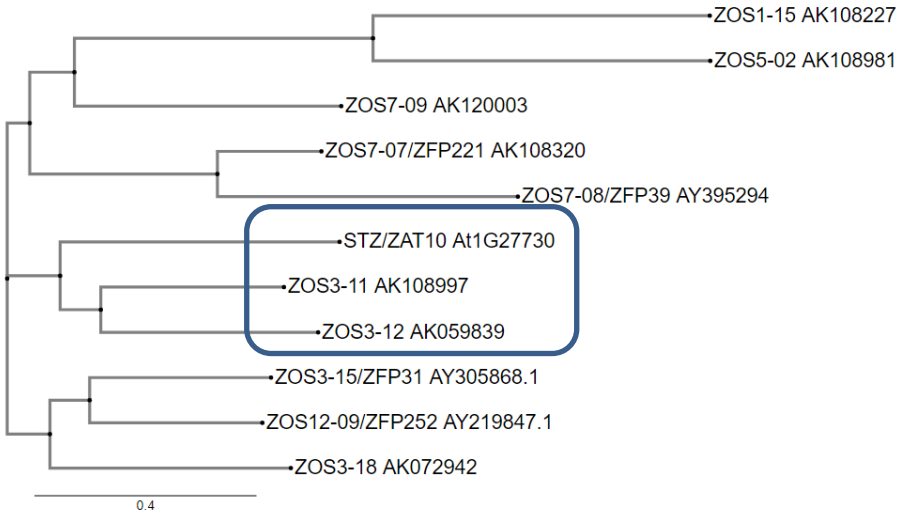
3.1.1 Sequence analysis of ZOS3-11 and ZOS3-12

Rice codes for 189 zinc finger proteins (ZFPs), which are classified in various types and distributes on all chromosomes. Among them 179 genes have been studied (Agarwal *et al.*, 2007; Englbrecht *et al.*, 2004). Different rice C2H2 transcription factors were selected based on various studies reported which were upregulated in salt stress. To know which ZFPs have close homology with STZ/ZAT10 of *Arabidopsis* which was induced by salt stress and could complement yeast calcineurin mutants and increase salt tolerance of transgenic yeast (Lippuner *et al*., 1996; Sakamato *et al*., 2000, 2004), phylogenetic tree was constructed using Neighbor-Joining method with the full-length amino acid sequence of all the selected proteins. The results revealed that the two transcription factors ZOS3-11 and ZOS3-12 in rice showed high homology with STZ/ZAT10 (Figure 3.1A). So, we selected ZOS3-11 and ZOS3-12 for our studies.

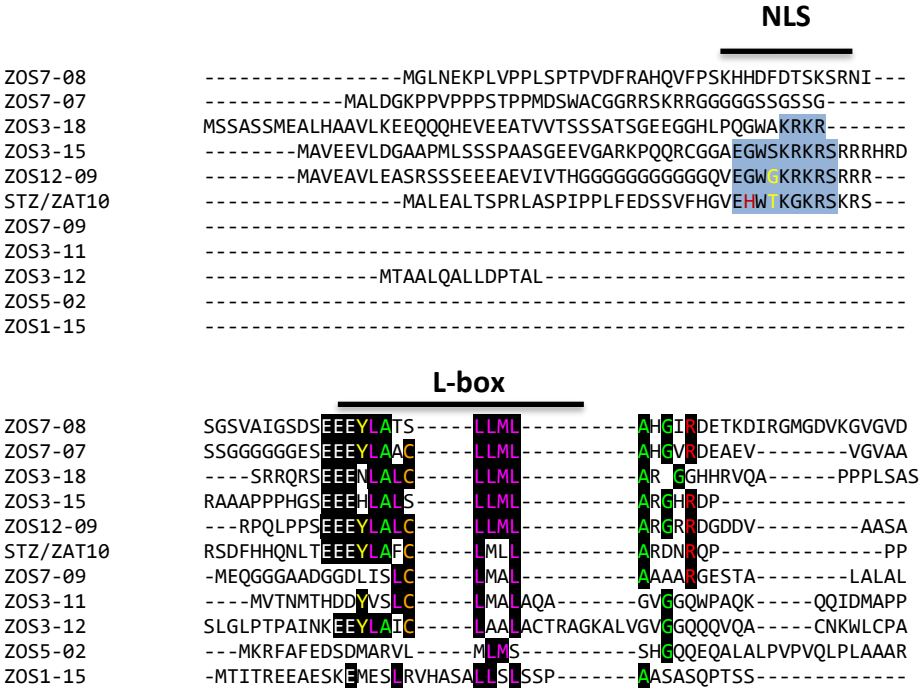
The length of the *ZOS3-11* cDNA sequence was 798 bp with an open reading frame (ORF) of 602 bp and *ZOS3-12* cDNA sequence was 1096 bp with an open reading frame (ORF) of 663 bp. They are located adjacently on chromosome 3. The ZOS3-11 and ZOS3-12 protein

contained 201 and 221 amino acids with a putative molecular weight of 21.35 kD and 22.8 kDa and an isoelectric point of 8.86 and 8.65. Clustal W alignment analysis indicated that ZOS3-11 and ZOS3-12 protein sequence contained two C2H2-type ZF domains. Each ZF domain contained a plant-specific QALGGH motif. Similar to most C2H2-type ZFPs in plants, ZOS3-11 and ZOS3-12 had a putative transcription repression domain, which was the so-called DLN-box (DLN) at the C-terminus, and an L-box (EEEYLALCLVML) motif related to protein-protein interactions but both lacks a B-box functioning as a putative nuclear localization signal (NLS) (Figure 3.1B).

A)



B)



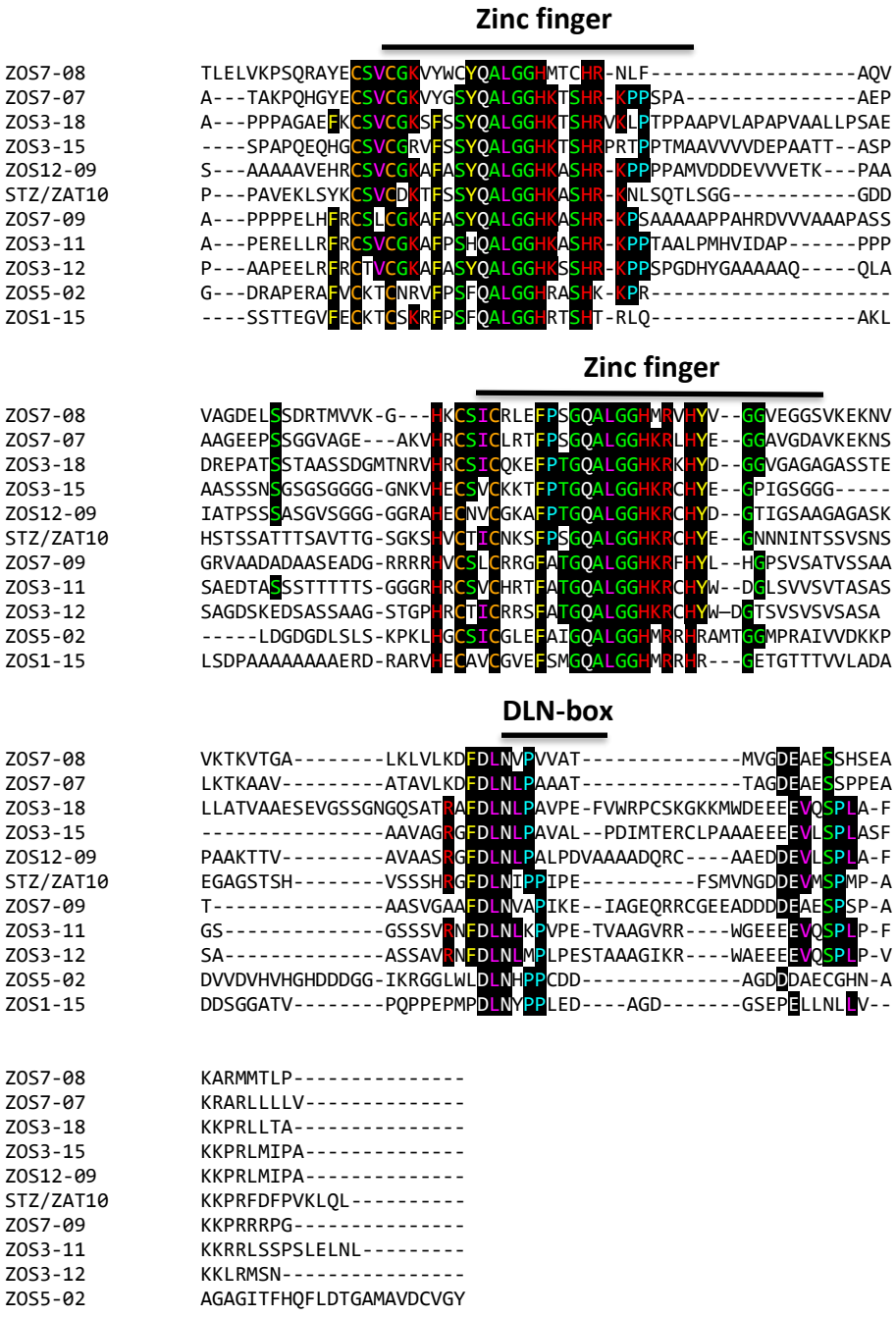


Figure 3.1: Checking evolutionary relationship of stress responsive C2H2 zinc finger protein in rice with STZ/ZAT10 of *Arabidopsis* (A) The phylogenetic tree of rice stress responsive C2H2-type zinc finger proteins and STZ/ZAT10 (name of protein along with accession number is shown). The close position of ZOS3-11 and ZOS3-12 and STZ/ZAT10 is highlighted in blue rectangle. The Neighbor–Joining tree was constructed with online software phylogeny.fr. (B) Multiple sequence alignment of

amino acid sequences of rice stress-responsive C2H2-type zinc finger proteins with STZ/ZAT10. The colored region indicates the positions at which the residues are identical. STZ/ZAT10 (Sakamato *et al.*, 2000), ZOS7-08(ZFP 245), ZOS7-07(ZFP221) (Huang *et al.*, 2005,2009), ZOS1-15(ZFP 179) (Sun *et al* 2010), ZOS12-09(ZFP252) (Xu *et al.*, 2008), ZOS3-15(ZFP31), ZOS5-02, ZOS7-09, ZOS3-11, ZOS3-12, ZOS3-18 (Agarwal *et al.*, 2007)

3.1.2 NLS lacking ZOS3-11 and ZOS3-12 are localized in the nucleus

In the multiple sequence analysis, the two proteins ZOS3-11 and ZOS3-12 lacked NLS (nuclear localization domain). To confirm the subcellular localization of these proteins, ZOS3-11/12-GFP fusion proteins were transiently expressed the under the control of the cauliflower mosaic virus (CaMV) 35S promoter in rice coleoptile (Figure 3.2) and BY-2 (Figure 3.3) cells and confirmed that the fusion protein specifically localizes to the nucleus even though they lacked NLS domain.

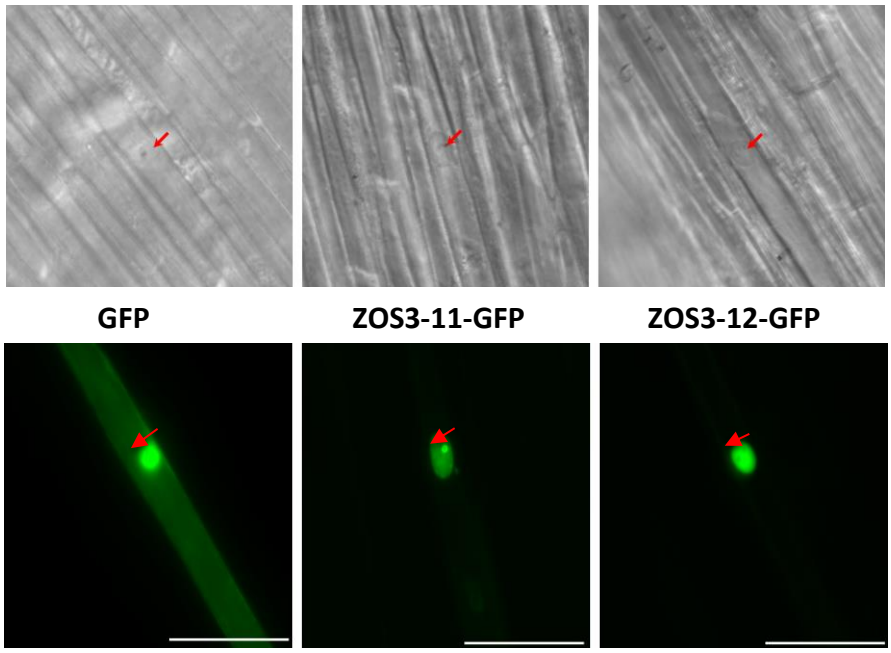


Figure 3.2: Localization of ZOS3-11 and ZOS3-12 in cells of rice coleoptile. GFP (Left) and the fusion constructs ZOS3-11-GFP (center) and ZOS3-12-GFP (right) were transiently expressed under the control of the CaMV 35S promoter. Shown are DIC (top) and fluorescence images (bottom) of each transformed cell 12-20 h after the biolistic transformation. The position of the nucleus is shown by arrow (scale bar represents 50 µm) (Result taken from Master thesis of René Glenz)

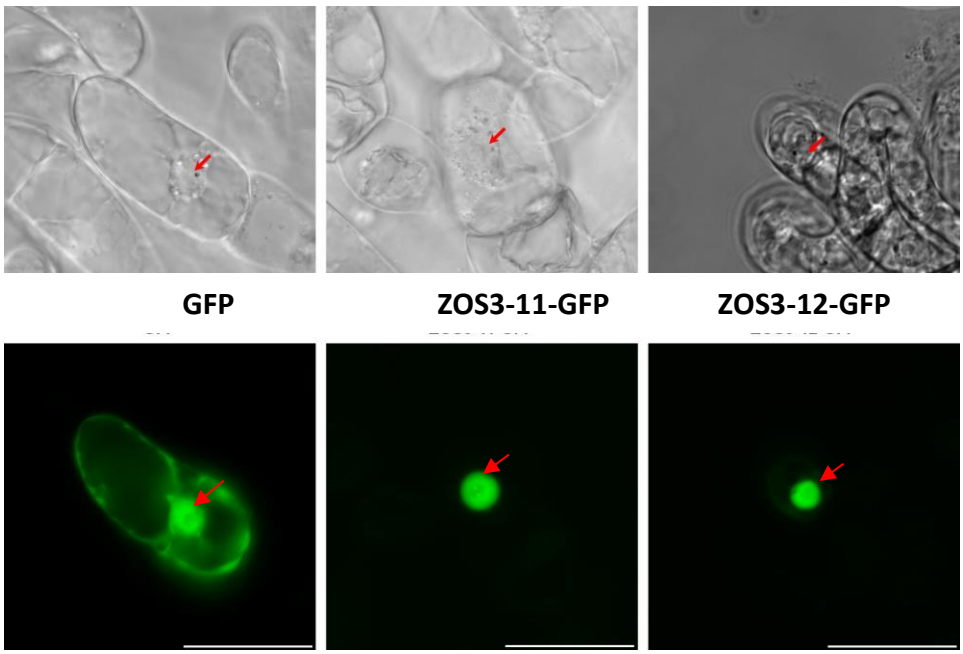
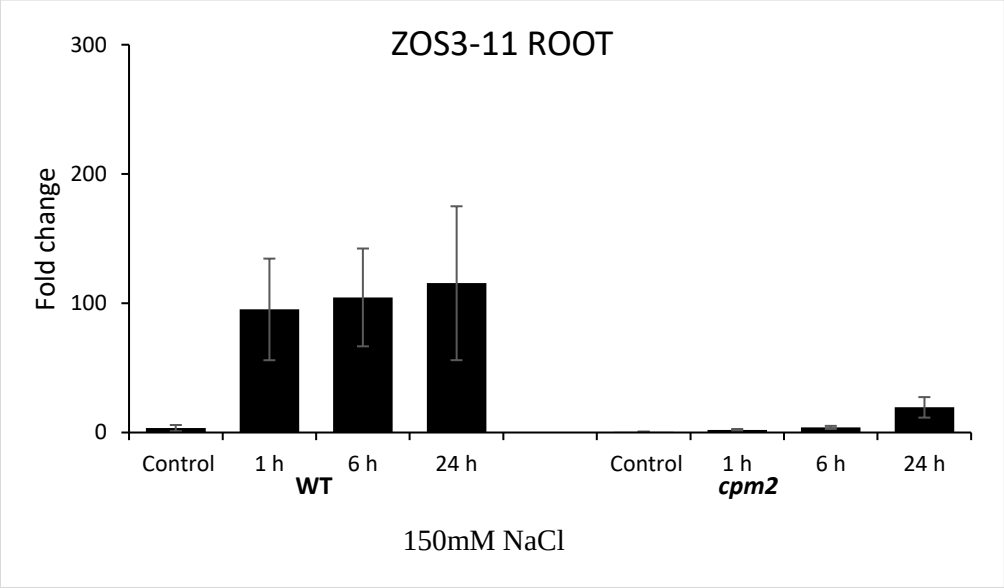


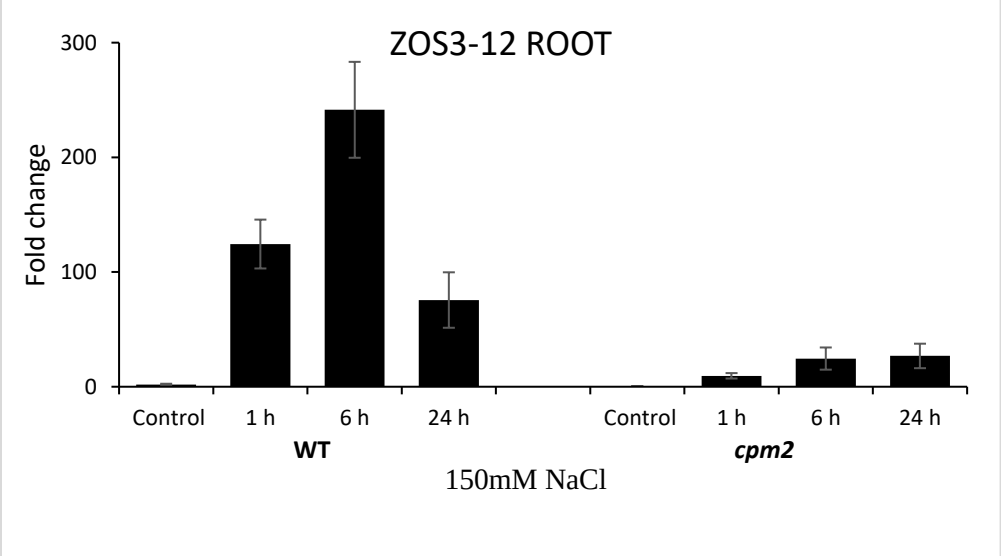
Figure 3.3: Localization of ZOS3-11 and ZOS3-12 in cells of BY2 liquid culture. GFP (Left) and the fusion constructs ZOS3-11-GFP (center) and ZOS3-12-GFP (right) were transiently expressed under the control of the CaMV 35S promoter. Shown are DIC (top) and fluorescence images (bottom) of each transformed cell 12-20 h after the biolistic transformation. The position of the nucleus is shown by arrow (scale bar represents 50 µm) (Result taken from Master thesis of Rene Glenz)

3.1.3 Expression of *ZOS3-11 and ZOS3-12* highly depends on JA but moderately on ABA

We tested whether the expression of *ZOS3-11* and *ZOS3-12* is regulated by salt stresses and ABA. Real-time PCR analysis revealed that the *ZOS3-11* and *ZOS3-12* transcript were highly expressed in roots, followed by leaves in case of *ZOS3-12*. There was no detectable amount of *ZOS3-11* in leaves. Up-regulation of *ZOS3-11*(100 fold) and *ZOS3-12* (200 fold) compared to the wild type control after 6 h of salt treatment in WT root was found and that the expression resumed continuously even at 24 h after the salt treatment in case of *ZOS3-11* but the expression for *ZOS3-12* decreased considerably after 6 h (Figure 3.4). On the other hand, ZOS3-12 was expressed comparatively less in the leaves when compared to root and the expression was higher at 24 h (30 fold) after treatment. The expression level of both proteins

was considerably lower in jasmonate mutant *cpm2*. This could mean that the expression level of these two proteins is highly dependent on jasmonate.





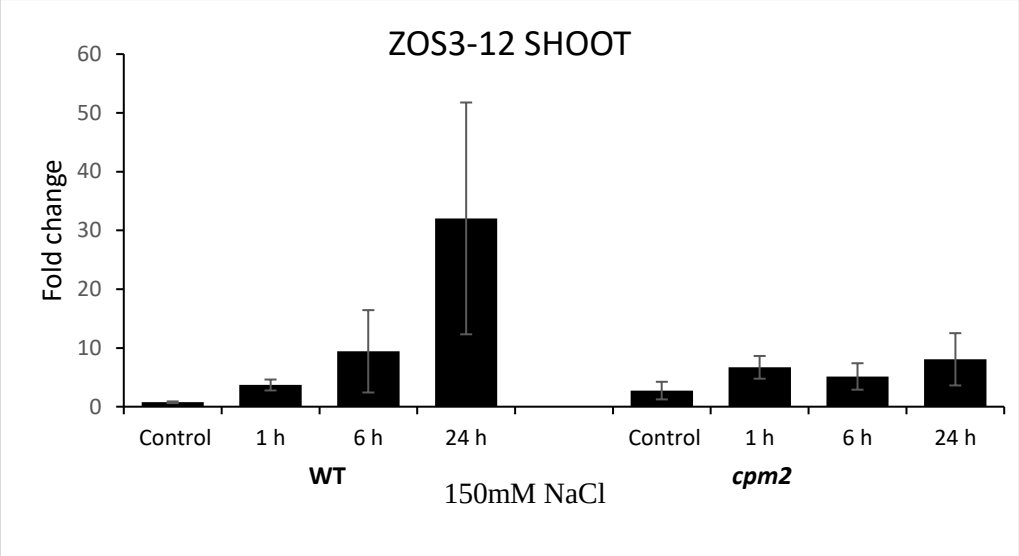
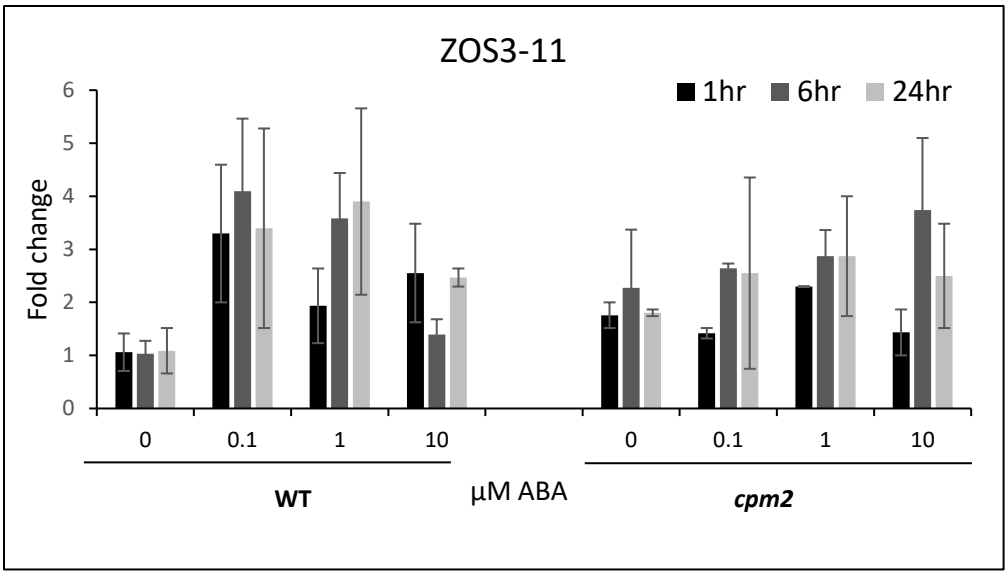


Figure 3.4: Relative gene expression analysis of *ZOS3-11* and *ZOS3-12* under salt stress. Rice wild type (Nihonmasari) and jasmonate mutant *cpm2* were treated with 150 mM NaCl solution or water (Ctrl). The samples were collected (1 h, 6 h and 24 h) after treatment. The relative amount of transcript was determined by normalization of the housekeeping genes EF-1α and UBQ5. Data represent average of three biological replicates with three technical replicates in each experiment. Error bars shows the standard error value. (Result taken from master thesis of René Glenz)

Real -time PCR analysis of root samples treated with ABA (0.1, 1 and 10 µM) showed moderately increased expression level for *ZOS3-11* and *ZOS3-12* and any considerable difference between the expression levels in WT and cpm2 was not found (Figure 3.5).



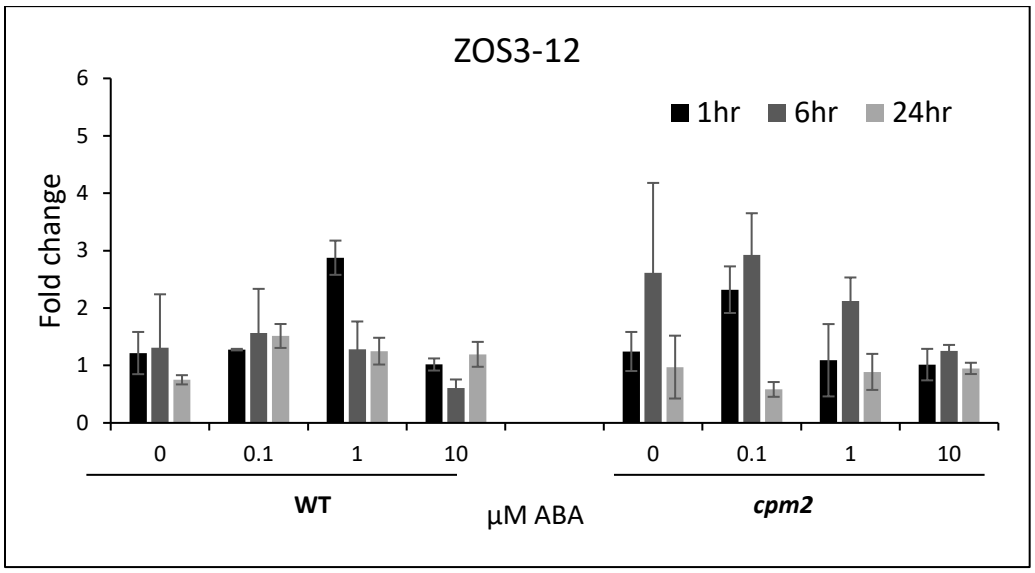


Figure 3.5: Relative gene expression analysis of *ZOS3-11* and *ZOS3-12* under ABA treatment in rice roots. Rice wild type (Nihonmasari) and jasmonate mutant *cpm2* were treated with 0.1, 1, 10 µM ABA solution or ethanol-water (Ctrl). The samples were collected (1h, 6h and 24h) after treatment. The relative amount of transcript was determined by normalization of the housekeeping gene UBQ5. Data represent average of three biological replicates with three technical replicates in each experiment. Error bars shows the standard error value.

3.1.4 Specific binding of ZOS3-11 and ZOS3-12 to A(G/C)T repeats

We adapted the DNA-Protein-Interaction (DPI)-ELISA technology (Brand *et al*., 2010) to find the target sequences or the cis-regulatory DNA sequences of ZOS3-11 and ZOS3-12. Constructs with histidine-tag for this assay is shown in Figure 3.6. In this method, different biotin-labelled DNA probes having all combinations of hexamer are bound onto streptavidin-coated 96-well plates; then, binding of ZOS3-11 and ZOS3-12 to DNA probes is detected by α-His-HRP antibody followed by HRP reaction. The proteins are extracted under native condition. The expression of purified recombinant histidine-tagged ZOS3-11 and ZOS3-12 are confirmed with SDS PAGE and western blot (Figure 3.7A), the probes which were positively bound by the protein in DPI-ELISA assay is shown in Figure 3.7B. Graphs were plotted based on the absorbance on the positive well in the y-axis and the positive probe number in the x-axis (sequences shown in the figure legend) (Figure 3.7C). The positively bound probes contained different ACT/ AGT sequences separated by certain number of nucleotides showing

that the ZOS3-11 and ZOS3-12 bind to these sequences. Both proteins showed similar result by binding to same probes and highest affinity was shown for the sequence where ACT and AGT were separated by 13 bp. This result was similar to three petunia ZPT2-related proteins, ZPT2-1, ZPT2-2, and ZPT2-4, which also bind to two copies of the AGT core sequence separated by 10 bp (Takatsuji *et al.*, 1994), the wheat WZF1 protein has been shown to interact with a DNA fragment containing tandem copies of a CACTC sequence (ACT box; Sakamoto *et al.*, 1993). As the ACT box has the AGT core sequence in the reverse orientation, it shows the importance of A(G/C)T for the binding of the ZOS3-11 and ZOS3-12.

To confirm the importance of A(G/C)T repeat sequences within the probe sequences for binding ZOS3-11 and ZOS3-12, the probe which showed the highest positive result for both the proteins was selected (Probe number 324 having sequence **Bio-AAAAA<u>ACT</u>GGATGCGCTCACC<u>AGT</u>AAAAA**) which contained ACT and AGT sequence separated by 13 nucleotides. Different probes were created by base substitution with mutating the ACT, AGT and both and also the nucleotides in between and the DPI-ELISA assay was repeated to show alteration in the binding capacity of the protein (Figure 3.7C), which confirms specific binding of the fusion protein to ACT and AGT and these sequences seem to be the core target site of ZOS3-11 and ZOS3-12 and also the sequences between ACT and AGT might also influence on the binding capacity (Figure 3.7C).

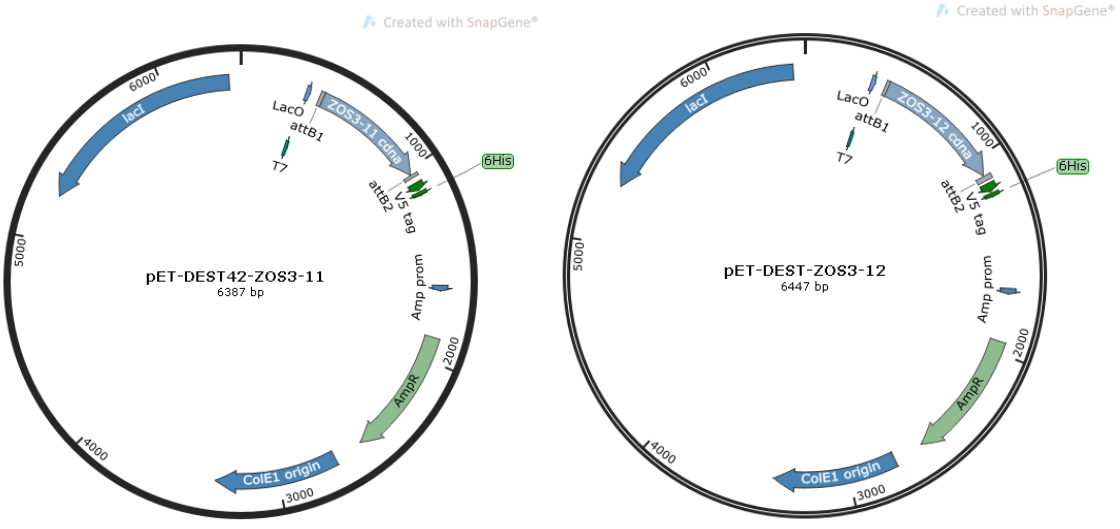
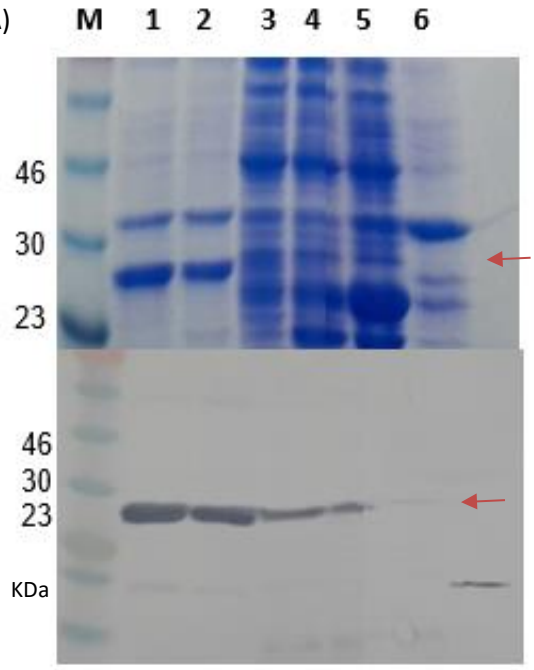


Figure 3.6: Expression vector pET-DEST42-ZOS3-11 and pET-DEST42-ZOS3-12 used for protein expression.

A)



B)

ZOS3-11

Top ranking

324_	Bio-AAAAA	CTGGATGCGCTCACCAGTTA AAAAA
14_	Bio-AAAAA	ACTCAACTAGTGAACCACC AAAAA
279_	Bio-AAAAA	CACTTAACTGAGTGGGATTG AAAAA
208_	Bio-AAAAA	GCTGTCACTGTAGTCGGTCC AAAAA
32_	Bio-AAAAA	CCTACGATGGTTCTGATCTT AAAAA
53_	Bio-AAAAA	AATACTGCGCACTTGCACTC AAAAA
108_	Bio-AAAAA	GGCGTCTGACTGTAACTCGT AAAAA

ZOS3-12

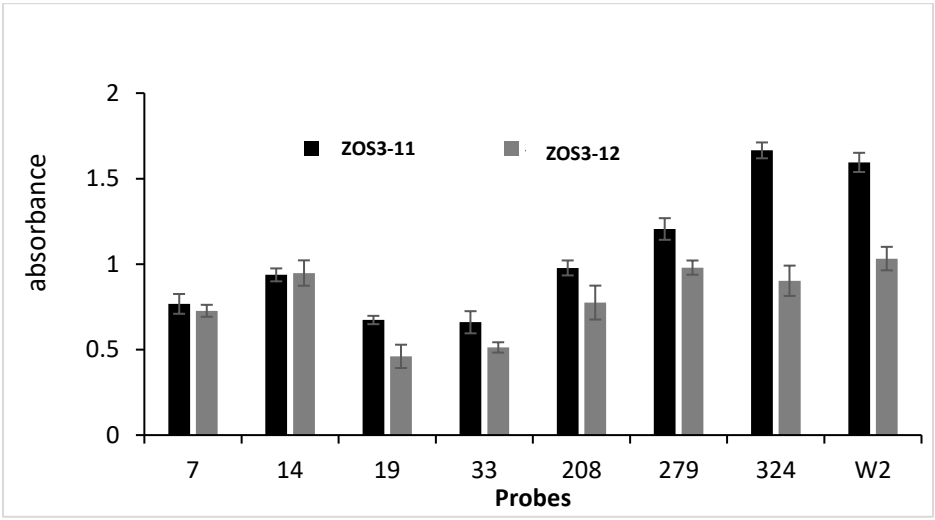
Top ranking

324_	Bio-AAAAA	CTGGATGCGCTCACCAGTTA AAAAA
14_	Bio-AAAAA	ACTCAACTAGTGAACCACC AAAAA
279_	Bio-AAAAA	CACTTAACTGAGTGGGATTG AAAAA
22_	Bio-AAAAA	TCTTCACCTCACTTCGGTTT AAAAA

Next ranking

321_	Bio-AAAAA	CTGGCGACTGCCAGTGGTAG AAAAA
291_	Bio-AAAAA	CGCCTTATGCCTCCCGCTCG AAAAA
208_	Bio-AAAAA	GCTGTCACTGTAGTCGGTCC AAAAA

C)



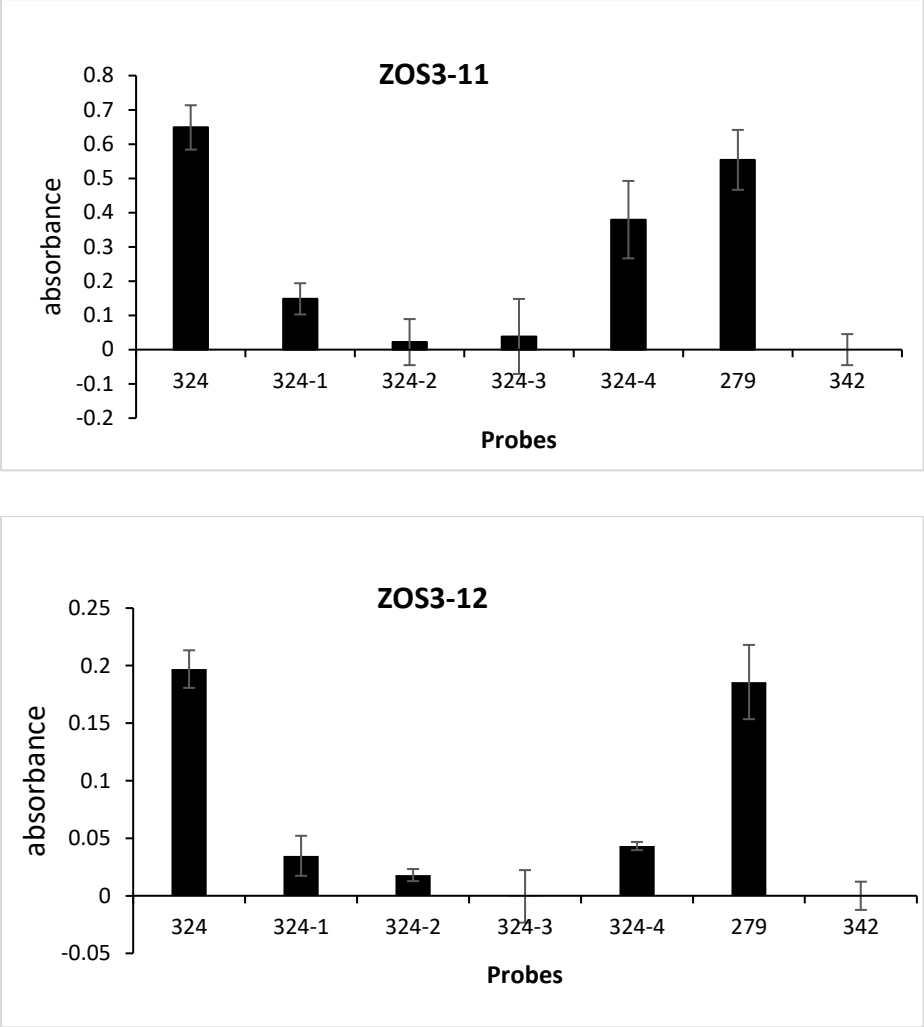


Figure 3.7: DNA-binding capacity of *ZOS3-11 and ZOS3-12.* **A**) Coomassie-stained SDS-PAGE gel (up) is shown for His-epitope tagged proteins and immobilized with anti-His-antibodies in the crude extract by western blotting using (down). Lane M-marker, 1-unsoluble ZOS3-11, 2-unsoluble ZOS3-12, 3-soluble ZOS3-11, 4-soluble ZOS3-11, 5and 6 -soluble and unsoluble part of empty vector, the region of protein band is shown by red arrow B**)** Positively bound probes in DPI-ELISA method. The colored letters show the ACT and AGT sequences present in the probes **C**) Absorbance results of *ZOS3-11 and ZOS3-12* binding to the positive probes (up) [**7**-AAAAAAGCTTCGCGCCAGCGGGAAAA, **14**-AAAAAAACTCAACTAGTGAACCACCAAAA, **19-** AAAAAATGAATCAATTTTTGGGCTTTAAAA, **33-**AAAAAATTTTGTTTTTGCTTTTCCTTAAAA, **208-** AAAAAAGCTGTCACTGTAGTCGGTCCAAAA, **279**-AAAAAACACTTAACTGAGTGGGATTGAAAA, **324**-AAAAAACTGGATGCGCTCACCAGTTAAAAA] and **W2**-AAAAAAATCAAAA GTTGACCAATAATAAAA as positive control. Middle and down graphs show the assay repeated with mutated probes for ZOS3-11 and ZOS3-12. Different probes used are **324- ACTN$_{13}$AGT**, **324-1 AaaN$_{13}$AGT**, **324-2 ACTN$_{13}$Aaa**, **324-3 AaaN$_{13}$AaT**, **324-4**

ACTS$_{13}$AGT, probe **279** as positive control and 343(-GGGGGGGGGGGGGGGGGGGGG GGGGGGGG) as negative control.

3.1.5 ZOS3-11 and ZOS3-12 confirms A(C/G)T binding and shows repression property

To assess the ability of ZOS3-11 and ZOS3-12 to act as a repressor in a more direct assay, we examined whether ZOS3-11 and ZOS3-12 were able to repress the *OsJAZ11* and *OsJAZ12* promoter in co-transfection assays. Given that the *OsJAZ11* and *OsJAZ12* promoter was activated in rice leaves by MeJA treatment, any repression effect of a transfected ZOS3-11 and ZOS3-12 expression vector might be expected. However, when rice leaves were transfected with a *OsJAZ11* and *OsJAZ12* promoter-luciferase reporter in the presence ZOS3-11 and ZOS3-12 expression vector, we were able to demonstrate significant repression by ZOS3-11 and ZOS3-12 in case of *OsJAZ12* promoter (Figure 3.8), but no significant difference in case of *OsJAZ11* promoter (data not shown). The degree of repression obtained could be increased by increasing the amount of effector (transcription factor). To justify this result, the promoter sequence of *OsJAZ11* and *OsJAZ12* (-1 to -1000 from start codon) was analyzed (Figure 3.9). It was observed that the *OsJAZ12* promoter contained more number (counted 9) of ACT-AGT or ACT-ACT sequence with distance less than 13 bp (this number was selected from DPI-ELISA result where one of the positive probe 324 had ACT and AGT separated with 13 bp and other positive probes has less distance (208, 279,14)) (Figure 3.7B). On the other hand, OsJAZ11 promoter had only 4 such sequences and was also in longer distance compared to in case of OsJAZ12 promoter. (Contructs used shown in Appendix 5.3).

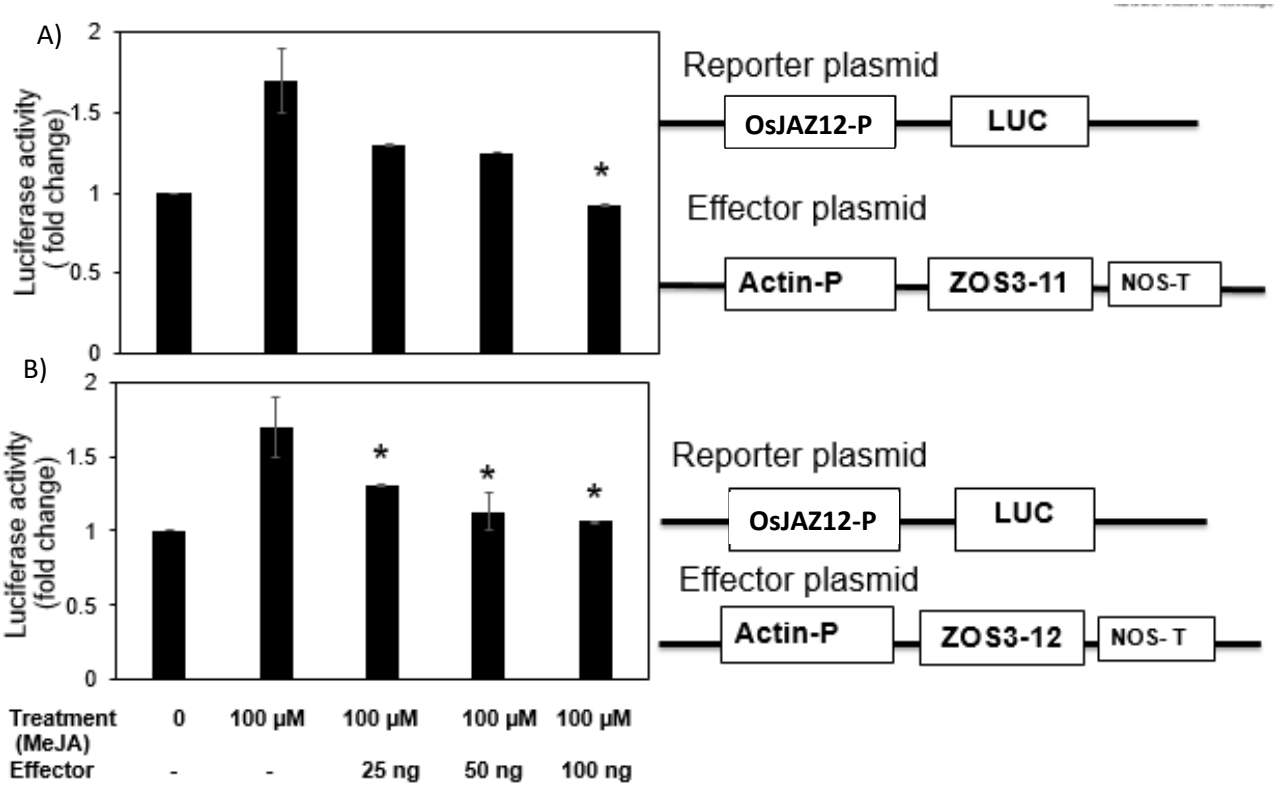


Figure 3.8: Lack of transactivation potential of ZOS3-11 and ZOS3-12 measured by Dual luciferase assay by OsJAZ12 promoter activity after MeJA treatment co-transformed with different concentrations of effector A) ZOS3-11 (25, 50, 100 ng/ml) B) ZOS3-12 (25, 50, 100 ng/ml). The data shows the fold change of luciferase activity of OsJAZ12 promoter in combination with different concentration of the effectors obtained after 24 h of 100 µM MeJA treatment compared to the control (treated with ethanol-H_2O). Right side of graph shows schematic diagram of reporter and effector constructs used in the transient assay. The effects were driven by Actin promoter (Actin-P). NOS-T represent polyadenylation signal of the nopaline synthase gene. The reporter gene LUC -luciferase was driven by OsJAZ12 promoter (OsJAZ12-P). Data represent averages of three biological replicates. Error bars shows the standard error value. The asterisk shows a significant difference compared with wild type ($P < 0.05$) by Student's t test)

A) OsJAZ11 promoter

CTCAGACTCAACGTGCACACGGGCGCACTTGCTTGACACGTTTCTTTACACACGATGGAGCATACA
TGAGGGCCCAAACGGTGCCCAAACCTAACCACCATGTGCGTGCGTGCGTGCGTACGTACTGTACA
TGCCCCGCTAGCCGTAGCCGGCCAGAGTAAACCAAAAATTAGGCCATGTGTAGTTTAGGGGTGA
AAAATTTTCACGAGTCACATCGGATATACATACACATATTTAAAAGCGTAGACTAATAACAAAAA
CAAATTACATATTCCGCTTGTAAAGTGCTAGACAAATTTATTAAGTATAATTAATCCGTCATTAAT
AAATATTTACTGTAGTACAATATTATCAAATCATATAGCAATTATGCTTAAAAGATTTGTCTCGTA
ATTTACACGTAATCTGTGTAATTTTTTTTATTTAATTACTCTATACATGTGACTAAACATTTAACGTG
ACAAGGTGAAAAGTTTTTACGTGGGAACTAGATAGGGCCTTTAACCGATAAGAGCAAGATTAATA
ATACAGCCAACTTACTGGCTATAAGGTTCTTTATAGTATTCTCTCAGCGTACCTTTATAATATAGTT
AGCTTTTTATCATTAATATTGGGTACACTTGCCTCTCTCACAGAGTTTCTTGGTTCTTATGTCCAAA
CCGGCTGTTAAGTTTACAGCTCGCTTCTCCTCTCTCTCCTCTCTTCTCTCCTCCACCTTATCATTTAG
CAGGCTTACTATTATATTTGCTCTAACCCAAAACCAACCAGACCATTTTCCCAGCGTTGTCATTTGC
CCCATTCGCACGCCACCACCACCGGTGGATTTACACCGCATCGCTGGCGCGGCCGCACGACGCAC
GTGATCCCCCCCCACGCAGCCACGCTGCGGAGGCAGAGGTAGCGCGCGCCGCCGCCGAAACAACCC
AACCCAACCCAACGTCCCGACCCGATCGAACAACCACCGCGAAAACAGCGAGAGACGTGCGGCG
CGTGCCCGCAATTTCTCGTCTTTTTTTCCGCGCGGGCGCCGCGCTTCCCTCCGCTCGCGTTTCACGT
GCCGCTTTCCTACCCGAGAAACAAAGATTATACTAGGCGTTTAGGCCTCGTCTTCCTTCCTTCCAC
ATGTACACCTAGTATATAACAAGAGGGAGACAGGCTACAGATTCACGGGAAGGTTTCGTCCTCGT
TTCGTTTCTTGCAGCAGGAAGCCAAAGTGTATTTCCCGAGCTTTGATGTGAGCTAGCTGATTTTGCT
AGAGCTACAACATG

B) OsJAZ12 promoter

TAAATATGCGCTATTTTTTTAATTTGTTTCTTCATGAGTTATTTACATTGGTTTGTTAGTGTTACATT
CAAGATGTTTTCTGCATGCTCTTCATCAGTTAATTATTGCATTCCGAATTCTAACCCTTTCTTTGAA
TTCAGAACAAGGTACGAATTGTGCTAGATTTATATCAGTTCGTAGATATTAAAATTAGGAAGACA
ATAAAAGTGTTAAAAATAATATACGTCAAGAAAAAATGTGTGCAATACCAATTTGAGCTTGTAAA
AATAGTAACTGACTAACTTTTATTATATTTATAGGTATTTTGGTCGTTTGGATAAAAATGTTAAGTA
CTAGGCGGAGGTTAACAGGACGGTGGTGGTAAATGTAGAAGTTAAGCAAAAATCGATGACATATT
ATAAAAAGTGACAAAGTCAGTGACAATCATAGACCTGTAACAAACTCAACTCAACGGTCAGCTGG
TGGTTCTACTCAAGGTGCGGGACACGAGAGCACAGTGTGAGGCATGACGGAGGCACGCACTCATG
AAGATGAAGTCGAGGCACAGATGGCGTATACACATAAAGTATACCATACCTGGGACATAATATCG
TGCCCTAGCAATCCTATGCATAGTATCCCACAACCAAAAAAAATCAATAAAGAAAAGATATTTTTT
TCTCTTGCAAATTAAGCAAAAATAATAAAATAGAAACAAAGGATCCACAGAGAGAGAGAGAGGC
CTAGACGAATTTTAGAAACCACAGGAAGATAAACCAAAGGAGCTAATGGCGTGTTTTAGTCCCTC
TAAAAATAGATAGATAGATAAACCAGTCGACGAGGTTCCAAGGAGAGAAGCGACAATTGTACTA
CTCCTCCCTCGCCCTCGCCGCCGCGCGTCGCTCTCCCTTCCCGCGCTCGCATTTCACGTGCTCCTTT
TCCAACCCAAAGCCAAAGGAAGCCCATCAGCTTAGCTATATAGCGCGCACCACCACTCGGCGTGT
ACACACACACACATCCCACCTCCCCACACGCAAGCTTCGCAGCGCAGCTTCGCGGAGACAACGTG
AGAAAGAAAGAAACGAGACGAACGCGAGGTGGTGGAGGTGGACGCCATG

Figure 3.9: Promoter sequence of OsJAZ11 (upper) and OsJAZ12 (lower) 1000 bp upstream of the start codon AGT which is highlighted in green. ACT and AGT sequences are highlighted in yellow and blue, respectively. The highlighted region represent the ACT/AGT sequence located close with the separation not greater than 13 bp.

3.2 Phenotypic studies in WT and transgenic BY-2 suspension cell lines (ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC) in different stress condition

3.2.1 Transgenic BY-2 cells showed better adaptation under salt stress

The generated constructs ZOS3-11::JAZ8 and ZOS3-12::JAZ8 containing *OsJAZ8* and ZOS3-11::JAZ8ΔC and ZOS3-12::JAZ8ΔC containing *OsJAZ8ΔC* under the control of salt inducible promoters ZOS3-11 and ZOS3-12 which were transformed into BY-2 cells and confirmed the expression of the transgene by RT-PCR (Appendix 5.8). The stable cell lines were used for further experiments. The constructs generated is shown in figure 3.10.

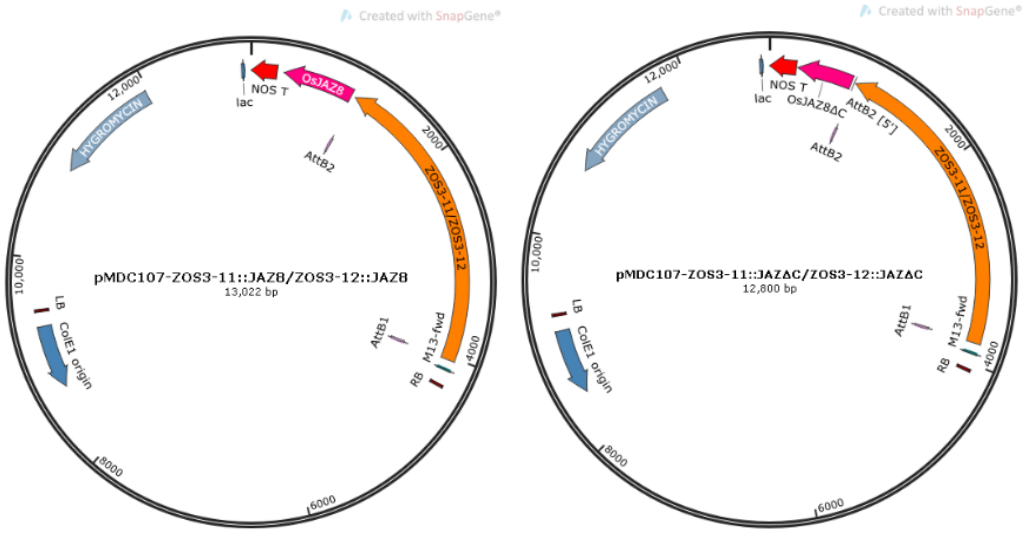
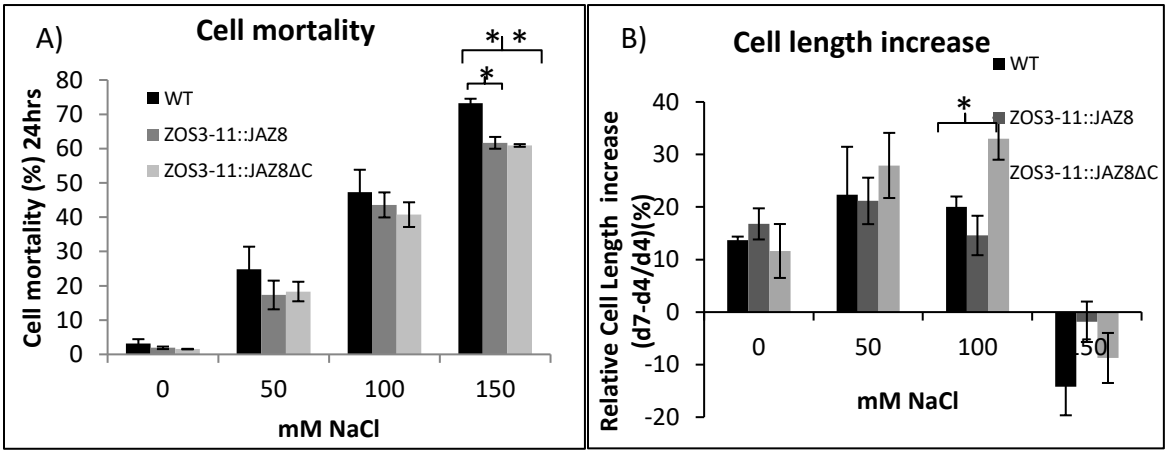


Figure 3.10: Plasmid constructs used for generating stable transgenic rice and BY-2 cell lines containing containing the TDNA inserts. RB-Right border, ZOS3-11/ZOS3-12 promoter region having attR1 and attR2 sites at both ends, downstream to promoter is the OsJAZ8/OsJAZΔC cDNA, NOS-T-nos terminator, Hygromycin antibiotic resistance, LB-left border.

To detect potential effects of salt and MeJA on the WT and the transgenic BY-2 cell lines, these were monitored by quantitative phenotyping, using cell viability, packed cell volume, cell elongation, and cell cycling time as parameters. There were no morphological differences between the transgenic and the WT BY-2 cells under normal growth conditions for both ZOS3-11 and ZOS3-12 promoters. When different parameters like cell mortality, packed cell volume

(PCV) or (biomass), cell size (increase in cell length in the stationary phase) and cell cycle duration were checked on the cells under stress conditions like salinity, no significant difference was found in case of ZOS3-12::JAZ8 and ZOS3-12::JAZ8ΔC lines , so here only the result of ZOS3-11::JAZ8 and ZOS11::JAZ8ΔC are shown as these lines showed a significance difference in the parameters analyzed.

When under salt stress, ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC showed 10% reduction in cell mortality rate at 150 mM salt compared to the WT, but no significant difference at 50 and 100 mM salt stress (Figure 3.11A). Approximately, 20 and 10 % increase in packed cell volume (biomass) at 50 and 100 mM salt stress was observed (Figure 3.11C). The cell cycle duration gives an estimation of time taken to double the number of cells. The doubling time can be detected from the cell density taken in time course manner based on the model of exponential growth. The results clearly show a longer time taken for WT cells to divide compared to the transgenic cell lines at 100 and 150 mM salt stress (Figure 3.11D). Cell elongation happens during stationary phase of the cell cycle. To determine whether salt affects in the cell elongation process, cell of the cells treated with different concentration of salt was measured during the start (4th day) and end of stationary phase (7th day) and relative increase of cell length was calculated. All the cell line showed increase in cell length at 50 mM then gradually decreased with increase in salt concentration, with the exception where a considerable increase (13%) in cell length was observed in case of ZOS3-11::JAZ8ΔC at 100 mM salt (Figure 3.11B).



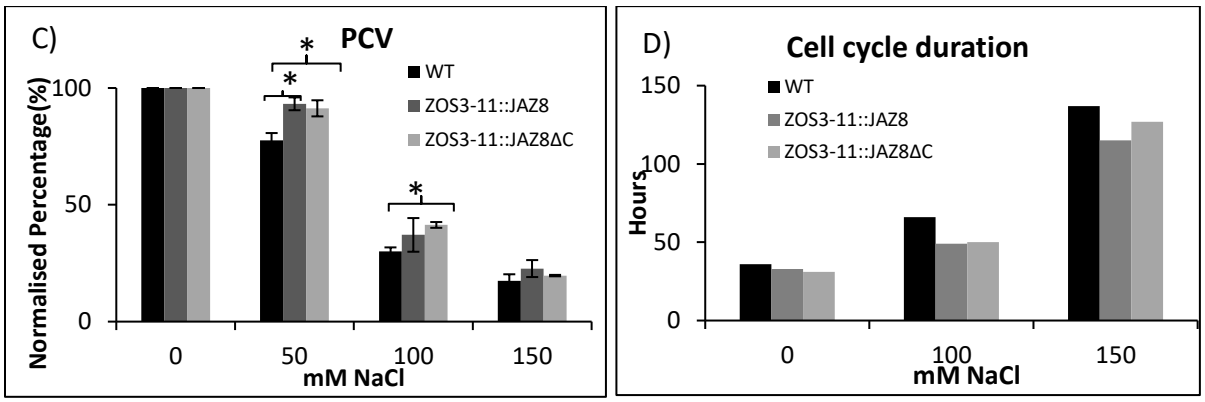


Figure 3.11: **Measurement of different parameters of WT, ZOS3-11::JAZ8 and ZOS11::JAZ8ΔC cells treated with series of salt concentration of 50 mM, 100 mM and 150 mM**. A) Cell mortality percentage B) relative cell length increase in the stationary phase (d4=day 4, d7=day 7). C) Packed cell volume D) Average cell cycle doubling time. Data represent average of three biological replicates. Error bars shows the standard error value. The asterisk shows a significant difference compared with wild type ($P < 0.05$) by Student's t test)

3.2.2 ZOS3-11 promoter was activated by MeJA but not by salt

In order to confirm whether the promoter was active in the transgenic cell lines, and knowing that the expression of *ZOS3-11* was highly regulated by jasmonic acid (Figure 3.4). The promoter is speculated to be induced by MeJA. To test the activity of the promoter in BY2 cells, the dual-luciferase reporter assay system was employed. Salt and MeJA treatment was given to BY-2 wild type suspension cells transiently transformed with pZOS3-11/pLuc and pZOS3-12/pLuc promoter-reporter system. And as speculated, compared to salt treatment, the promoter showed increased activity (2 fold) in case of MeJA (Figure 3.12).

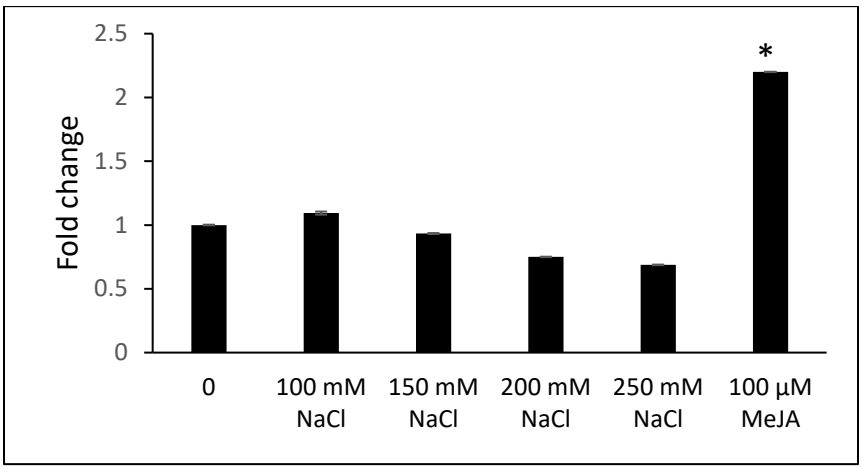


Figure 3.12: Dual luciferase assay for measuring ZOS3-11 promoter activity after salt and MeJA treatment. The data show the fold change of luciferase activity of ZOS3-11 promoter obtained after 24 h after of 100, 150, 200 and 250 mM of salt and 100 µM MeJA treatment, three biological replicates. Error bars shows the standard error value. The asterisk shows a significant difference compared with wild type ($P < 0.05$) by Student's t test)

3.2.3 MeJA treatment shows jasmonate insensitive phenotype in transgenic BY-2 cells

Since the promoters showed enhanced activity under MeJA treatment (Figure 3.12), the same experiments performed in section 3.2.1 with salt were repeated with 100 µM MeJA. ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC showed significant reduction in cell mortality rate compared to the WT (Figure 3.13A) and showed reduction in cell cycle duration (Figure 3.13D). Cell density decreased tremendously in all the cell lines and transgenic cell lines showed no significant difference when compared with WT (Figure 3.13C). Interestingly, there was 44 and 65 % increase in cell length in ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC as compared to 20% in WT in the stationary phase (Figure 3.13B). This clearly confirms our hypothesis of the transgenic cells showing jasmonate insensitivity.

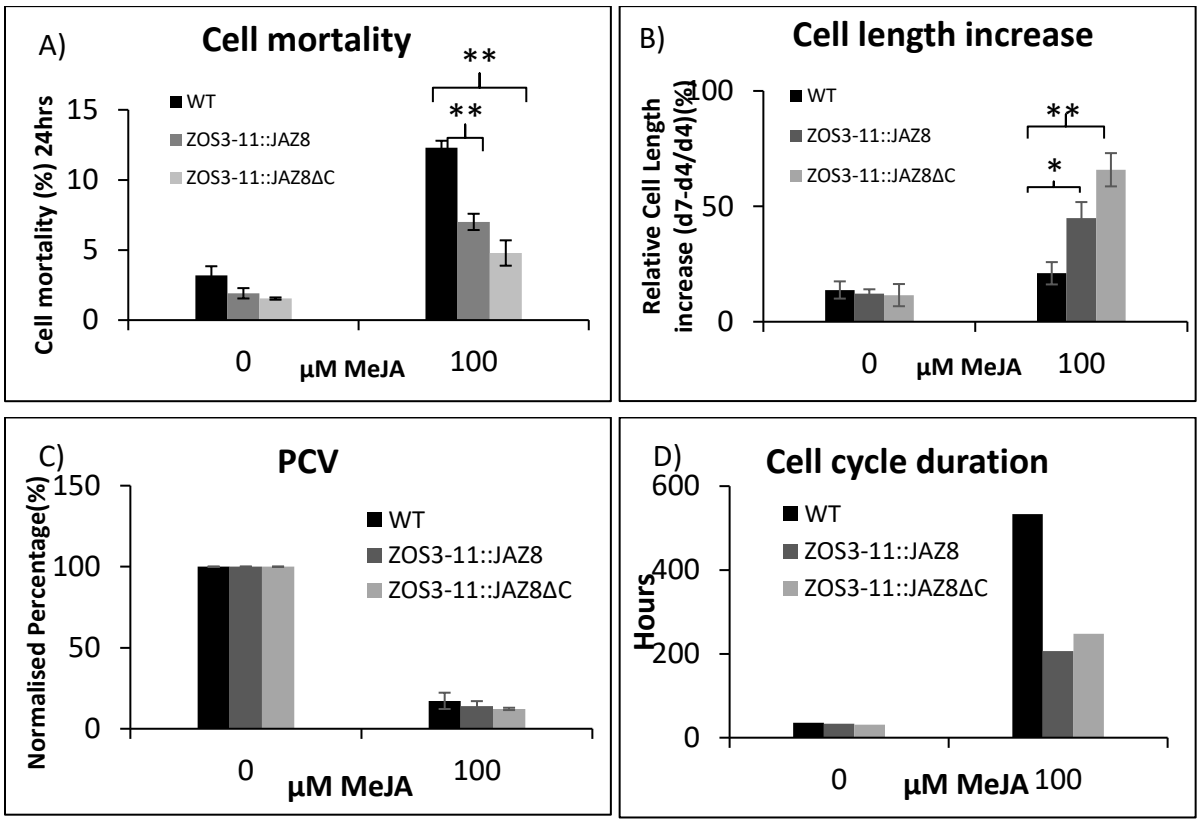


Figure 3.13: Measurement of different parameters of WT, ZOS3-11::JAZ8 and ZOS11::JAZ8ΔC cells when treated with 100 µM of MeJA a) Cell mortality percentage b) relative cell length increase in the stationary phase c) Packed cell volume d) Average cell cycle doubling time. Data represent average of three biological replicates. Error bars shows the standard error value. The asterisk shows a significant difference compared with wild type ($P < 0.05$) by Student's t test)

3.2.4 Transgenic BY-2 cells shows increased expression of *OsJAZ8* and *OsJAZ8ΔC* in response to MeJA

To confirm the overexpression of *OsJAZ8* in ZOS3-11::JAZ8 and *OsJAZ8ΔC* in ZOS3-11::JAZ8ΔC, we extracted RNA from the cell culture at an interval of 1 h, 6 h and 24 h after treatment with 150 mM NaCl and 100µM MeJA and compared the gene expression patterns. We performed three biological replicates for each treatment. The expression was observed to be highly induced in response to MeJA when compared to salt treatment in the ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC. The highest expression level was detected at 6 h, approximately 15-

fold in case of *OsJAZ8* and 17-fold for *OsJAZ8ΔC* respectively and was decreased at 24hrs (Figure 3.14).

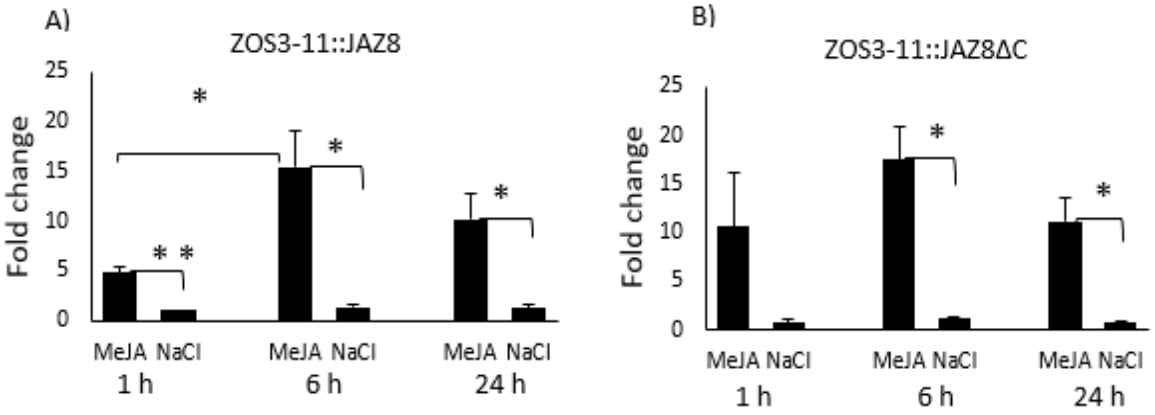


Figure 3.14: Relative gene expression of A) *OsJAZ8* transgenic BY-2 lines ZOS3-11::JAZ8 and B) *OsJAZ8ΔC* in ZOS3-11::JAZ8ΔC. The fold change was calculated compared to the respective control in response to 150 mM NaCl and 100 µM methyl jasmonate. Data represent average of three biological replicates. Error bars shows the standard error value. The asterisk shows a significant difference compared with wild type ($P < 0.05$) by Student's t test)

3.2.5 Auxin responsiveness in the transgenic cell lines

In order to understand the growth stimulation in the transgenic cell lines compared to the WT, we assayed the auxin sensitivity and responsiveness of cell length increment (Figure 3.15). When the dose response curve of relative cell length increase was determined, the amplitude of the response was found to be dramatically elevated depending on the auxin concentration. This was especially impressive as growth in the WT was less induced, whereas it proceeded at almost the maximal velocity in the transgenic cell lines especially ZOS3-11::JAZ8ΔC. In contrast, the threshold and the maximum of the curve were reached at the same concentrations of auxin (3µM IAA) as in the WT. Thus, there are no indications for an increase of auxin sensitivity but shows amplified responsiveness in the transgenic cell lines in response to auxin which cleary confirms the insensitivity to jasmonate response in the transgenic cell line specially in ZOS3-11::JAZ8ΔC.

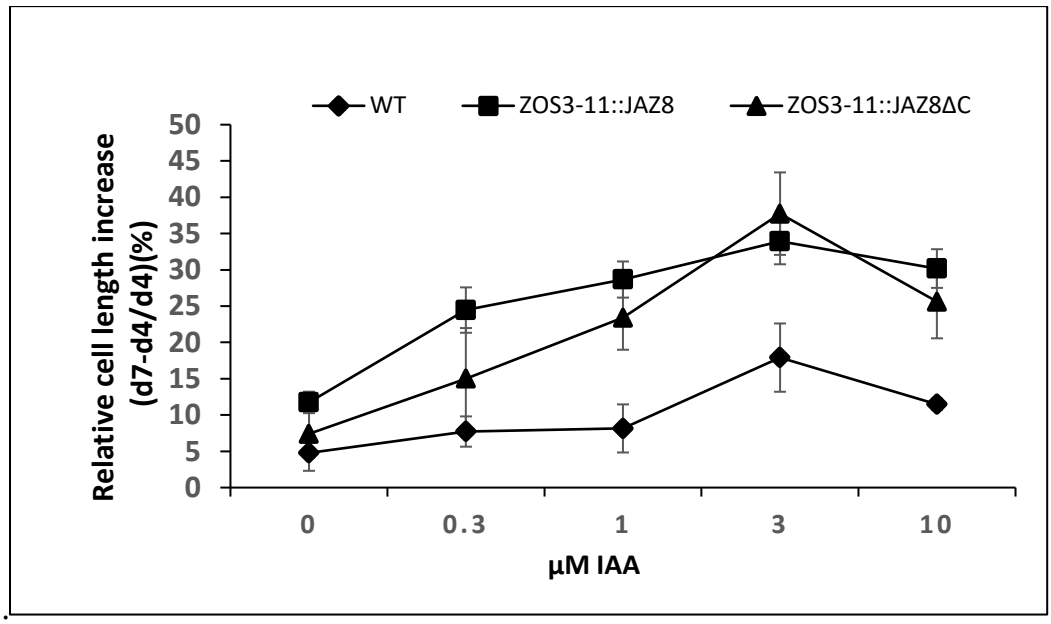


Figure 3.15: **Auxin response in WT and jasmonate insensitive BY-2 transgenic cell lines (ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC).** The values represent the relative cell length increase of 7 d old cells after IAA treatment with 4 d old cells before IAA treatment. Data represent average of three biological replicates. Error bars shows the standard error value.

3.3 Transgenic rice showed better tolerance to salt stress in the early stages

OsJAZ8 and *OsJAZ8ΔC*-overexpressing rice plants under the control of salt inducible promoter ZOS3-11 was generated by *Agrobacterium*-mediated transformation. Independent lines of the second or third generation after transformation were used for further experiments. Experiments were carried out to check the performance of the transgenic rice under 100 mM salt stress. Due to the availability of a limited number of seeds, only preliminary experiments could be performed at the moment.

Under no stress condition, there were not many morphological differences between the transgenic rice plants and the wild-type (WT) (Figure 3.16). Percentage of healthy seeds in each variety was compared to any difference in the yield of WT and the transgenic lines and

the ZOS3-11::JAZ8ΔC was found to have 15% increase in yield while ZOS3-11::JAZ8 showed 20% increase (Figure 3.17).

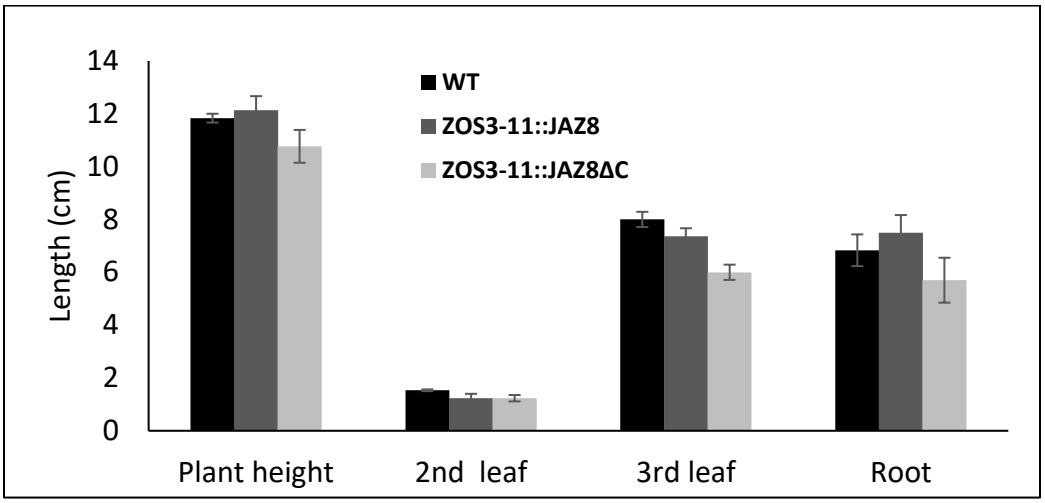


Figure 3.16: **Phenotype studies on WT and transgenic rice ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC** A) Length of shoot (upper), 2nd leaf (middle), 3rd leaf (lower) under no stress condition.

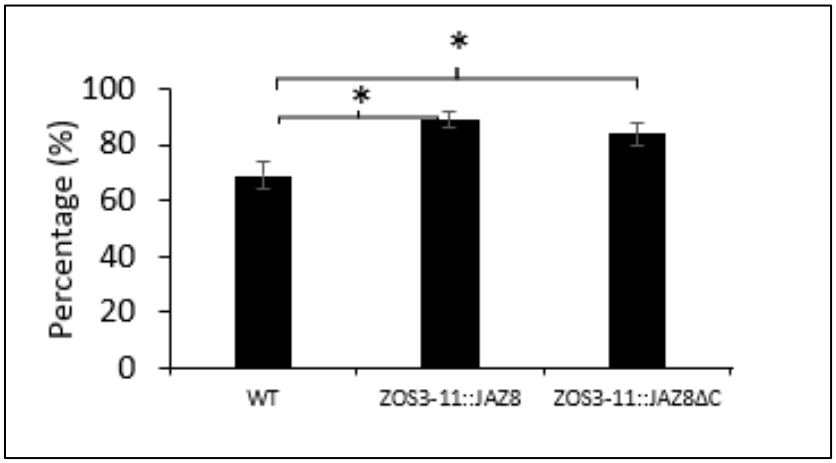


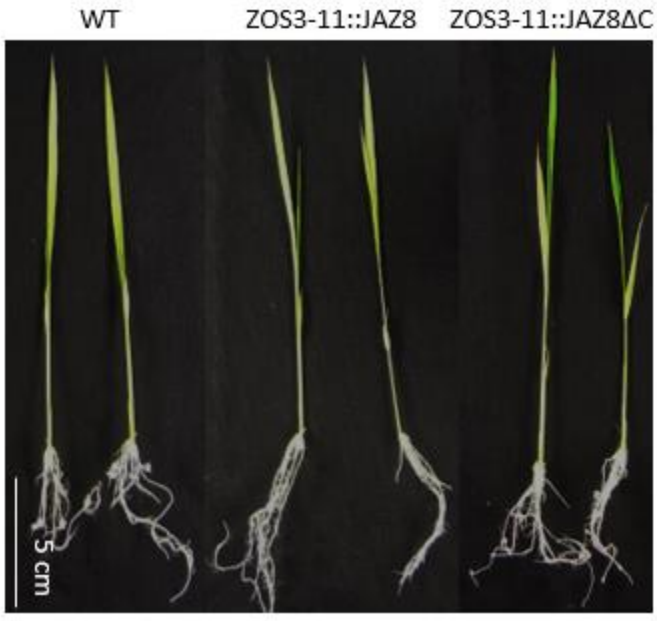
Figure 3.17: Comparison of yield of WT and transgenic rice ZOS3-11::JAZ8, ZOS3-11::JAZ8ΔC and ZOS3-12::JAZ8. It shows the yield in percentage (ie, number of good seed to total number of seed in one plant). Data represent average of three biological replicates. Error bars shows the standard error value. The asterisk shows a significant difference compared with wild type ($p < 0.05$) by Student's t test)

3.3.1 Transgenic rice showed improved salt tolerance under salt stress condition

To check our hypothesis whether dominant suppression of jasmonate will lead to salt stress tolerance, 10 days old seedling of the WT and the transgenic lines were treated with 100 mM salt and observed for three days. The relative increment in length of plant height, 2nd leaf (fully developed), 3rd leaf (developing) and root were calculated from length before and after 2 days of the salt treatment (Figure 3.19). The whole shoot, the 3rd leaf, and the root did not show any considerable increase in length nor it showed significant difference with each other. However, the fully developed 2nd leaf of ZOS3-11::JAZΔC showed a significant 50% increase in length compared to the other two.

The pictures were taken before and on 2nd and 3rd day after the salt treatment (100mM). Two days after the salt treatment clearly showed detrimental effects like leaf rolling and tip necrosis which was enhanced on the WT leaf compared to the transgenic rice leaf (Figure 3.18B). On the other hand, both the transgenic rice plants were clearly showing better performance by minimal leaf rolling and necrosis on the tip of the leaf. On the third day, the leaves of WT and ZOS3-11::JAZ8 were fully rolled and yellow, while the ZOS3-11::JAZ8ΔC showed necrosis in top one fourth part of leaf, but still the remaining parts were green and showed more tolerance to salt than the other two (Figure 3.18C). This clearly proves our hypothesis of salt tolerance under the condition of dominant jasmonate suppression.

A) No Stress



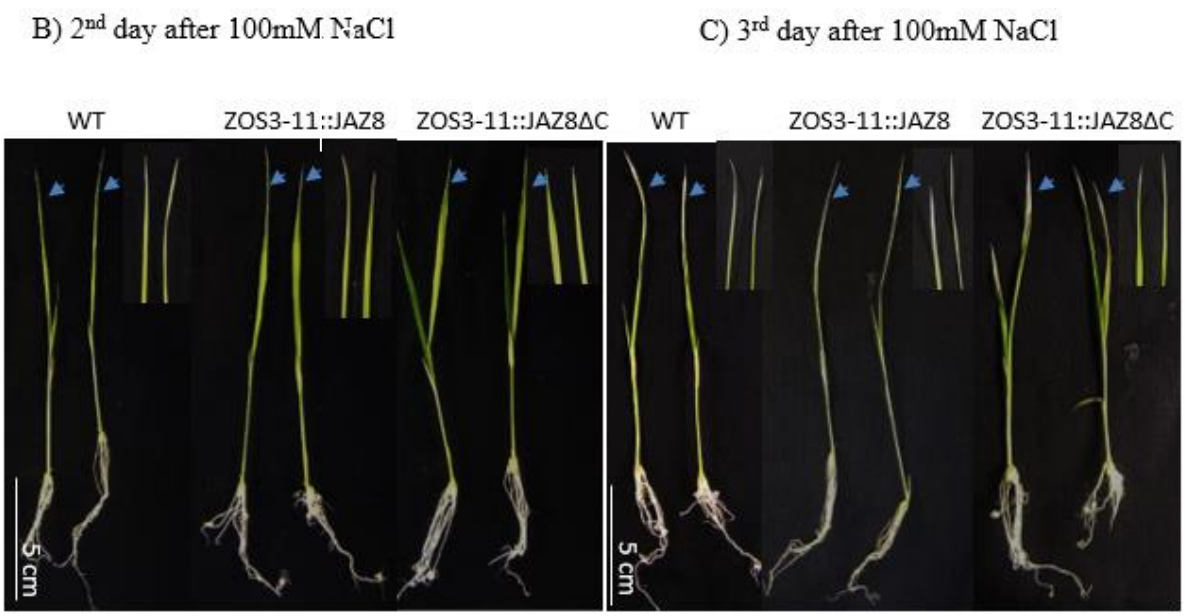


Figure 3.18: Observation of rice plants (WT, ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC) under salt stress. 10 days old rice plants were subjected to 100mM salt stress (A) Plants under control condition (B) Plants after 2 days (C) Plants after 3 days.

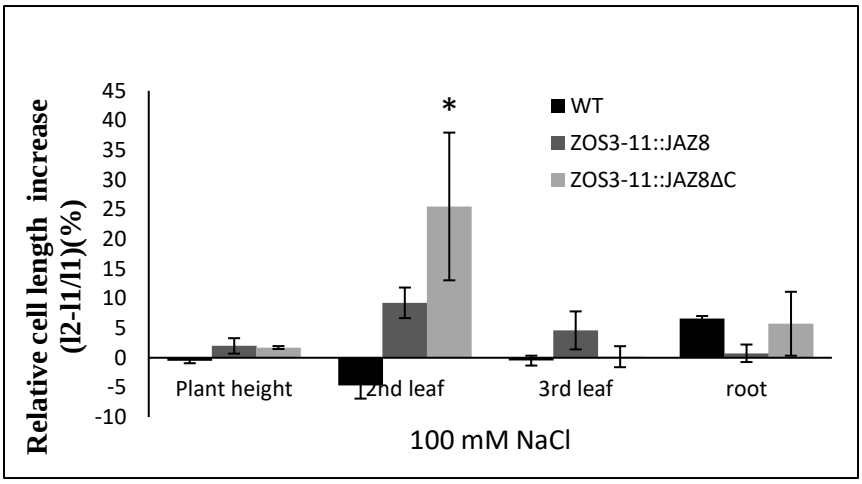


Figure 3.19: Relative increase in length of shoot, 2nd leaf (middle), 3rd leaf (lower) and root measured 2 days after 100 mM salt treatment. l2=length after salt treatment, l1=length before salt treatment. Data represent average of three biological replicates. Error bars shows the standard error value. The asterisk shows a significant difference compared with wild type ($p < 0.05$) by Student's t test)

3.3.2 High expression of *OsJAZ8* in the transgenic cell lines in response to salt stress

When the transcript level of *OsJAZ8* were compared, in the transgenic rice lines, the relative expression levels of the *OsJAZ8 and* gene were significantly greater in the transgenic lines than in the WT. On average, the transgenic lines showed 5- to 10-fold the expression of WT control and treated plants (Figure 3.20). The transgenic control plants had increased basal level of *OsJAZ8* compared to the wild type. When treated with 100mM NaCl, the highest expression level was detected at 6 h, approximately 15-fold in case of *OsJAZ8* and 20-fold for *OsJAZ8ΔC* respectively and was decreased at 24 h.

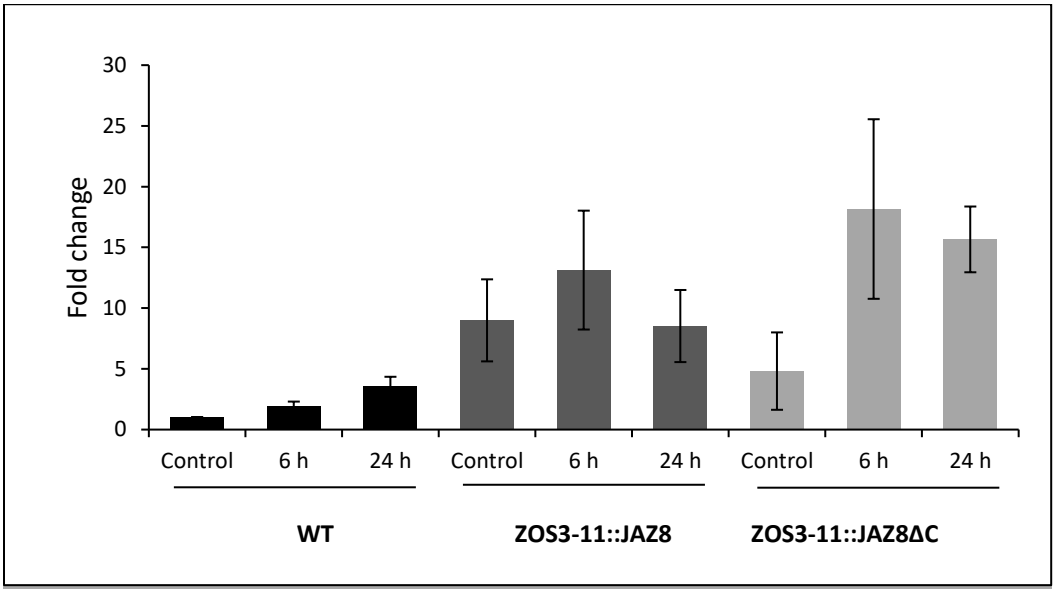


Figure 3.20: Relative gene expression of *OsJAZ8* in WT and transgenic rice leaves in response to 100 mM salt relative to WT control. Data represent average of three biological replicates. Error bars shows the standard error value.

3.4 Extended investigation on levels of precursors and catabolites of jasmonate and sodium uptake study over the time in rice plants exposed to salt stress

The previous results by Hazman *et al*., 2015 showed that JA-Ile precursor OPDA was accumulated under salt stress in the WT rice and no significant difference in JA content compared to the control. Since only one time point of 6 h was included here, we wanted to know the fate of JA, JA-Ile, OPDA in a time course along with the content of degradation products of JA-Ile (12-OH-JA-Ile and 12-COOH-JA-Ile). When measured these metabolites under salt stress of 100 mM over a three-time course of 6 h, 24 h and 72 h, the result showed not only increased accumulation of not only the OPDA, JA ad JA-Ile over time under but also the JA-Ile catabolites 12-OH-JA-Ile and 12-COOH-JA-Ile. Even though all the metabolites were accumulating until the end of time course, 12-COOH-JA-Ile was accumulated strongly compared to others (Figure 3.21). In all the samples examined, the content of metabolites in controls are always minimal.

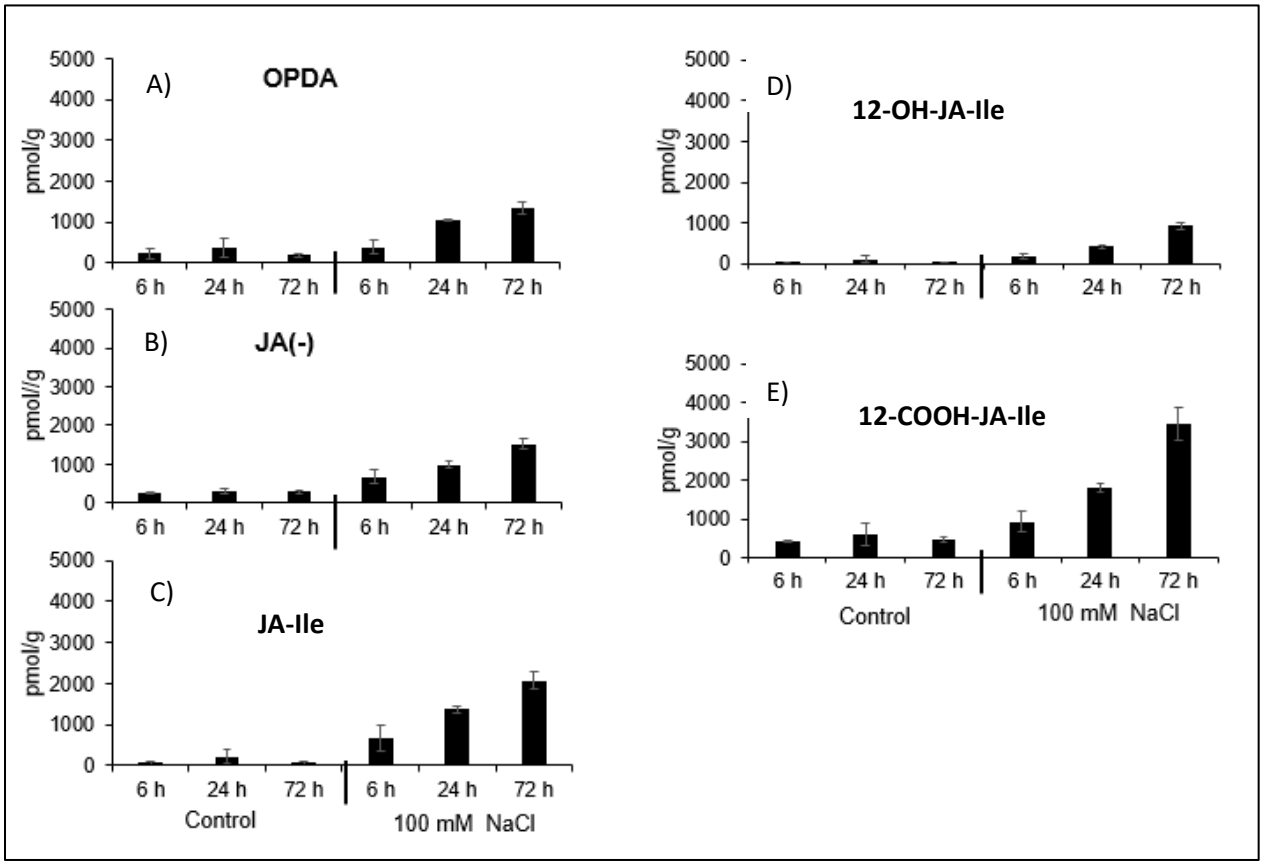


Figure 3.21: Results of hormone analysis A) OPDA B) JA C) JA-Ile D) 12-OH-JA-Ile and E) 12-COOH-JA-Ile data obtained by UPLCMS /MS performed on leaf samples of Nipponbare sampled at 6 h, 24 h and 72 h under control condition and salt stress of 100mM. Data represent average of three biological replicates. Error bars shows the standard error value. This result has been taken from Sandra Schäfer's Master thesis.

In the study of (Hazman *et al.*, 2015) on the effect of salt stress on the jasmonate mutants *cpm2* and *hebiba*, the mutants were shown to have better performance in salt stress However, these observations were based on experiments in a very young stage in which stress was applied for a short period. To elucidate more about the uptake of salt in the *jasmonate mutants cpm2 and hebiba*, we investigated the Na^+ and K^+ content in the shoot (4^{th}, 5^{th} and 6^{th} leaf) after cultivation in medium containing 100 mM NaCl for one week in WT(Nihonmasari), *cpm2*, *hebiba*, FL478 (salt-tolerant variety) and RC222 (salt-sensitive variety). A marked increase in Na^+ uptake was observed in the leaves of salt treated plants compared with the control plants (results not shown). Na^+ content in the control plants was negligible. There was a significant difference in Na^+ content between the 5^{th} and 6^{th} leaves of the wild type and the mutants on 7^{th} day after salt treatment (Figure 3.22A). It was observed to have lower accumulation of Na^+ in the 5^{th} and 6^{th} leaf of *cpm2* and *hebiba* and the Na^+ was mostly concentrated in the 4^{th} leaf causing leaf yellowing and necrosis (pictures shown in Figure 3.23) which was a phenomenon similar to the salt-tolerant variety FL478, indicating that one reason of having enhanced salt tolerance in *jasmonate* mutants might be due reduction of Na^+ transport into the younger leaves at the top of the plant. WT showed similar Na^+ uptake to salt-sensitive variety RC222 where Na^+ content was almost evenly distributed in 4^{th}, 5^{th} and 6^{th} leaves. K^+ content decreased in salt affected rice plants compared with the non-stressed control plants. K^+ content in the $4^{th,}$ 5^{th} and 6^{th} leaves of the WT and RC222 were decreased after salt stress compared to the control condition. On the other hand, FL478, *cpm2* and *hebiba* showed less K^+ in their 4^{th} leaf compared to the control but there was no difference in their 5^{th} and 6^{th} leaves (Figure 3.22B) which was in accordance with their high Na^+ in 4^{th} leaf which could lead to high Na+/K+ ratio in their 4^{th} leaf leading to their detrimental fate. This difference in salt distribution and accumulation in WT, *cpm2* and *hebiba* showed WT behaving like the salt sensitive variety

while *cpm2* and *hebiba* showing more resemblance to the tolerant variety and using this phenomenon to adapt to salt stress.

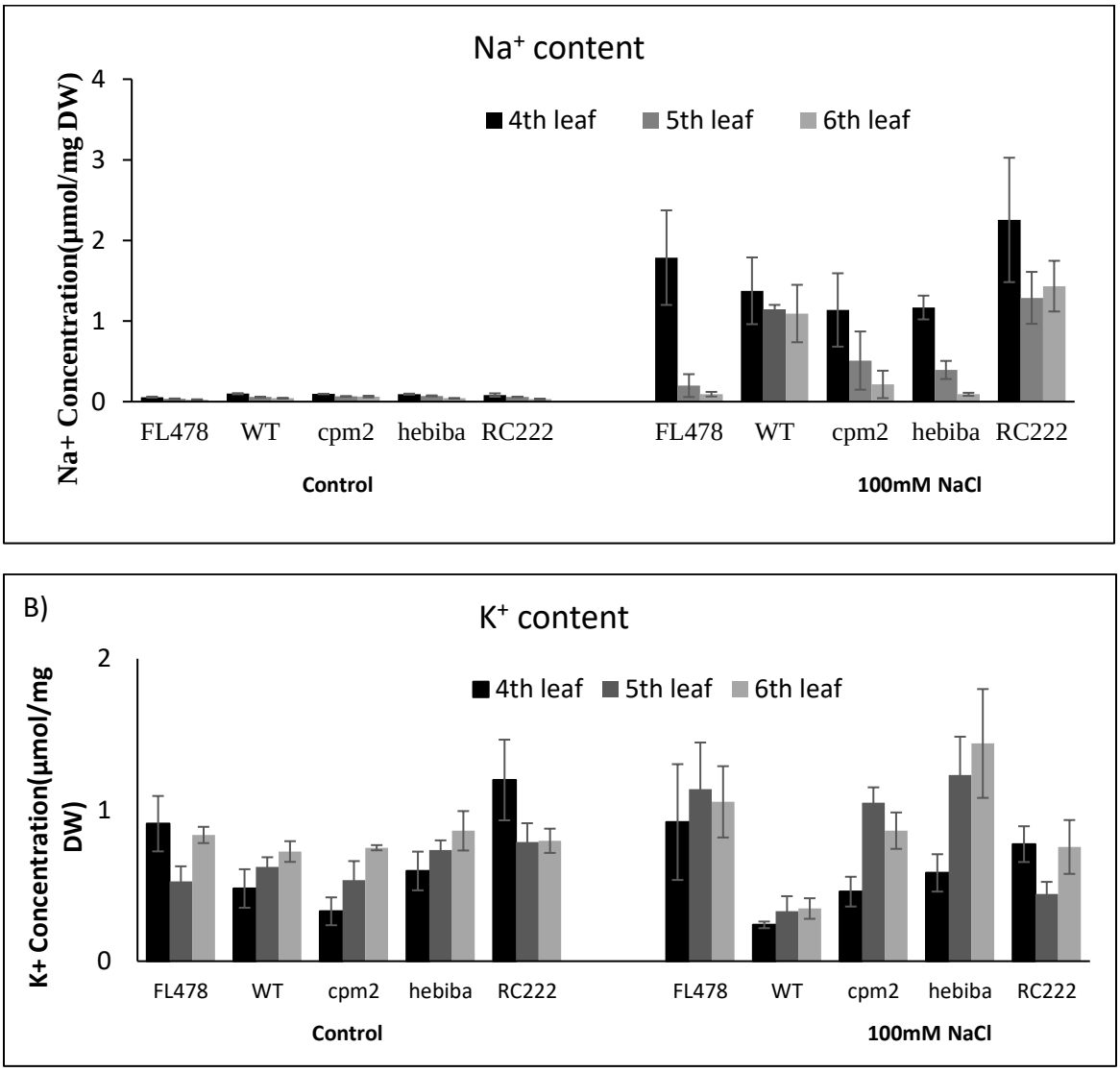


Figure 3.22: Content of A) sodium ion (Na^+) and B) potassium ion (K^+) in 4th, 5th and 6th leaf of WT(Nihonmasari), *cpm2*, *hebiba*, FL478 (salt-tolerant variety) and RC222 (salt-sensitive variety) on 7th day of 100 mM NaCl treatment. Values represent Mean of three biological replicates ±SD.

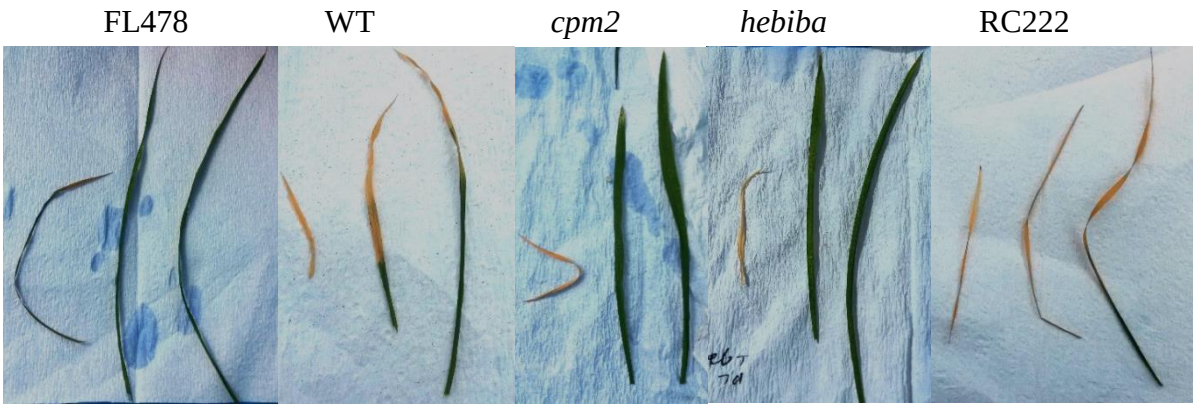


Figure 3.23: Effect of 100 mM NaCl on 4th (left), 5th (middle) and 6th (right) leaf of WT(Nihonmasari), *cpm2*, *hebiba*, FL478 (salt-tolerant variety) and RC222 (salt-sensitive variety).

3.5 Summary

This study was mainly intended to develop a strategy based on JA-sensitivity for obtaining plants which could suppress jasmonate signaling under high salinity avoiding the disadvantages of a general loss of JA signaling. Final goal of this approach is to establish such a mechanism in rice plants. Here we mainly used a heterologous system tobacco BY-2 cells for the experiments, and successfully established rice lines, for which first salinity assays could be carried out.

To design the plasmid constructs for this purpose, a JAZ repressor i.e., OsJAZ8, was selected based on previous reports. Full length and the jas domain (C terminal) truncated OsJAZ8 was used for the experiment to suppress the jasmonate signaling. This JAZ repressor was driven by promoter of C2H2 type zinc finger transcription factors (ZOS3-11 and ZOS3-12), respectively which was selected based on the fact that the transcription factor showed high homology with salt-induced C2H2 zinc finger transcription factor in Arabidopsis STZ/ZAT10. This construct having hygromycin antibiotic resistance was transformed into rice and BY2 cells for further investigation and to prove our hypothesis.

The data obtained in this study showed the following observations:

1. The phenotypic investigation revealed that the transgenic BY-2 lines ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC showed better performance by showing jasmonate insensitivity under different stress response of salt and MeJA by showing reduced cell mortality, increased cell length, reduction in cell cycle duration and increase in auxin responsiveness compared

to the non-transformed BY-2 cells. The response to salt stress was minimal compared to MeJA and this result was consistent with the activity of promoters where the promoters were activated with MeJA but not with salt. Using the transgenic tobacco lines as a model system, we found that the system enables one to monitor the activity of the promoter and induction of *OsJAZ8* and *OsJAZ8ΔC* gene leading to jasmonate insensitive phenotype thereby showing better performance in salt and MeJA treatment.

2. Preliminary results suggest that transgenic rice plants ZOS3-11:: JAZ8ΔC performed better under salt stress proving the dominant suppression on the jasmonate signalling, on the other hand in ZOS3-11:: JAZ8, the even though the plant behaved better initially, had detrimental effect on the next day, proving that the JAZ8 degradation in the later stages .

In parallel, ZOS3-11 and ZOS3-12 transcription factors were characterized, to find out that these protein are preferentially localized to the nucleus despite of a lacking nuclear localization signal. These proteins are highly induced by salt and the induction depends on jasmonic acid. The proteins were minimally induced by ABA. The Dual-luciferase assay showed that they have transcriptional repression activity and binds to ACT/AGT nucleotide sequence which was be proved by DPI-ELISA method.

As an approach to unravel more details about salt tolerance mechanism of the jasmonate mutants *cpm2* and *hebiba*, the level of accumulated Na^+ ions were measured using flame photometer. Sodium ion uptake was measured in 4^{th}, 5^{th}, and 6^{th} leaf. Interestingly, the younger leaves (5^{th} and 6^{th}) of the mutants showed less uptaken sodium ions than WT and most of the sodium ions were accumulated in 4^{th} leaf of *cpm2* and *hebiba*. This trend was similar to adaptation mechanism of salt-tolerant variety FL478 and the wild type showed sodium ion uptake trend similar to the salt-sensitive variety RC222.

The profile of hormonal production in response to salt stress was obtained in a time-course experiment. Jasmonates (JA, JA-Ile), its biosynthesis intermediates (OPDA) and degradation products (12-OH-JA-Ile and 12-COOH-JA-Ile) were analyzed in rice (Nipponbare) leaves and all the products were increased under 100 mM salt stress in a timely manner and accumulated most at 72 h after the treatment. The data indicate that salt stress leads to a continuous increase (biosynthesis) of jasmonates.

4 DISCUSSIONS

The role of phytohormone jasmonates in salinity adaptation are widely studied nowadays. However, it is difficult to make a direct correlation between jasmonate activity and salinity adaptation due to partially contradicting results. Some previous work in rice suggested reduction or inactivation of jasmonate results in salt tolerance, but these plants also exhibited detrimental effects for example, jasmonate mutants of rice *cpm2* and *hebiba* showed increased salt tolerance compared to their wild type (Hazman *et al.,* 2015), but exhibited male sterility (Riemann *et al.,* 2003, 2013). Similarly, inactivation of JA by overexpression of JA catabolizing enzyme CYP94C2b leads to salinity tolerance but also causes some detrimental effect on the plant (Kurotani *et al.,* 2015). However, on the other hand, unconstrained JA signaling also confers damage-related events that have to be controlled at the earliest to acquire adaptation to salinity (Ismail *et al.,* 2014). So, in order to negate the negative effect of jasmonate on plants and to enhance salt tolerance, suppression of the jasmonate response at the earliest is required there by a change in the temporal signature of jasmonate production and signaling could be obtained to increase salt stress adaptation.

To attain suppression of jasmonate signaling, one aspect is to overexpress jas domain truncated JAZ genes (*JAZΔC*). This concept was proved in *Arabidopsis,* where JAZΔC failed to be recognized by COI1, which prevented degradation after jasmonate perception, resulting in constitutive repression of MYC2 and therefore confer the jasmonate-insensitive phenotype (Chini *et al*., 2009, Chung&Howe 2009). Among several JAZ genes present in rice, we selected OsJAZ8 and Jas domain-truncated version or C terminal truncated (OsJAZ8ΔC) for this experiment based on a previous study by (Yamada *et al.* 2012), where overexpression of OsJAZ8ΔC caused JA-insensitive phenotypes in the transgenic rice plants., resistance to inhibition of root growth and inhibition of JA-induced chlorophyll reduction. These results indicate that expression of OsJAZ8ΔC inhibits JA responsiveness via the dominant-negative activity of the altered OsJAZ8 protein. They also demonstrated that OsJAZ8 formed heterodimeric complexes with some JAZ proteins but did not homo-dimerize in yeast, suggesting the possibility that OsJAZ8 may function as a heterodimer with other JAZ proteins to repress JA signaling in rice. In our study, we have tried to maintain the suppression of jasmonate signaling only under salt stress by driving these two

versions of *OsJAZ8* genes under the control of salt induced promoters ZOS3-11 and ZOS3-12 which are also regulated by jasmonates (Figure 3.2). Stable transgenic rice and transgenic BY-2 cells were prepared. Since it takes considerable time to obtain the transgenic rice suitable for the experiment, initials set of experiments were performed with transgenic BY-2 cells.

4.1 Suppression of JA signaling in transgenic BY-2 cells leads to JA insensitive phenotypes under salt stress and MeJA treatment

Under salt stress, transgenic cells showed better adaptive phenotypes such as reduced cell mortality, increased biomass, increased cell elongation during stationary phase and reduction of cell cycle duration at a higher salt concentration of 100 and 150 mM compared to the WT BY2 cells (Figure 3.11). These observations proved that the OsJAZ8 and OsJAZ8ΔC although expressed only in small amounts under these conditions (Figure 3.14) may help the cells to shift the negative effect of jasmonate in response to high salinity towards adaptation. This was in agreement with studies where constrained JA accumulation and signaling correlated with a precondition to escape salinity-induced cell death and to activate salinity adaptation (Ismail *et al.,* 2014a).

However, MeJA treatment (100 µM) clearly showed jasmonate insensitive phenotype in the transgenic cell lines by increase in cell length in the stationary phase specially in ZOS3-11::JAZ8ΔC (Figure 3.13). These properties are consistent with *Arabidopsis* JAZ proteins lacking the C-terminal Jas region which were resistant to COI1-dependent degradation after jasmonate treatment, and that dominantly showed jasmonate-resistant phenotypes such as resistance to JA-induced inhibition to root elongation (Chini *et al.,* 2007, Thines *et al.,* 2007, Yan *et al.,* 2007, Chung *et al.,* 2008).The ZOS3-11 promoter did not show activation under salt stress but was active with MeJA which proved the difference in response of cells to salt and MeJA. This difference in result can be correlated with the expression pattern of the *OsJAZ8* and *OsJAZ8ΔC*, which was highly expressed by MeJA but not by salt (Figure 3.14).

Explanation of the cell elongation of the transgenic BY-2 cell lines ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC under MeJA (Figure 3.13B) can be given from previous studies which reported that treatment with MeJA promotes the production of auxin (Uppalapati *et al.,* 2005) and MeJA increases *YUC8* and *YUC9* gene expression (*YUCCA* genes are involved in IAA synthesis) leading to increased IAA level and COI1-dependent signaling pathway are also involved in regulation of these genes (Hentrich *et al.,* 2013). The suppression of jasmonate signaling in the transgenic lines may confer to increase in IAA activity leading to cell elongation. To know more whether cell elongation in the transgenic lines have any connection to auxin, we have done IAA treatment in varied doses which is explained in the below section.

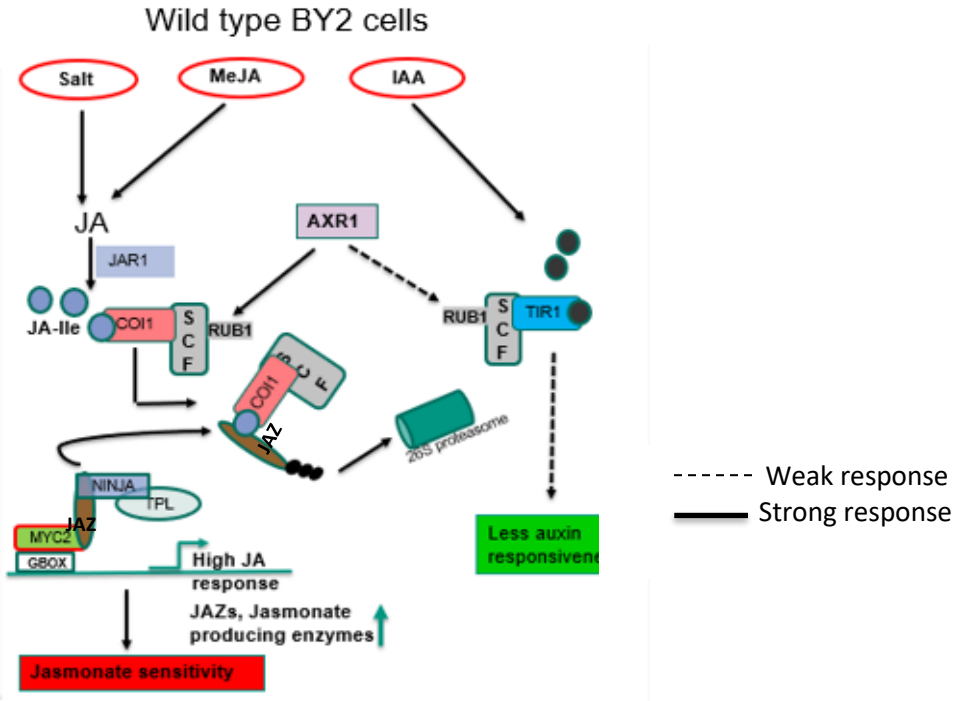
4.2 Suppression of jasmonate signaling alters auxin responsiveness in transgenic BY-2 cells

It is well known that plant hormones like auxin and jasmonates are tightly interconnected to fine-tune plant growth, development, and interaction the with environment. To confirm whether the result of increase in cell length of the transgenic BY-2 cell lines has any relation with auxin, dose response curve of relative cell length increase was determined. Interestingly, increased responsiveness to auxin was seen in the transgenic BY-2 lines ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC by increase in cell length, and the effect was higher in the ZOS3-11::JAZ8ΔC where the jasmonate signaling should be dominantly suppressed. To explain this phenomenon, one has to consider that on the level of hormone perception and signaling, both IAA and JA-Ile share same components like SCF E3-ligases and also same upstream regulator like AUXIN RESISTANT1 (AXR1), as mutations in these causes impaired responses to both hormones (Tyryaki & Staswick, 2002; Nick, 2006) and shared co-repressor like TPL (Szemenyei *et al.,* 2008; Pauwels *et al*., 2010). In a study by (Hentrich *et al.,* 2013) shows that two *YUCCA* genes (*YUC8* and *YUC9*) which lead to the production of IAA are overexpressed under MeJA treatment and causes inhibitory effects of MeJA on root elongation in *yuc8* and *yuc9* mutants. Auxin and JA pathways are also linked through the action of auxin response factors (ARFs) which regulate auxin-dependent genes as well as transcription of JA biosynthetic gene (Nagpal *et al.,* 2005). There were also reports showing their antagonistic properties as high JA causes reduction of accumulation of auxin transporters PIN1 and PIN2,

impairing auxin transport (Sun *et al.*, 2011) and also JA could act as auxin inhibitors by conjugating to amino acid Tryptophan (Staswick, 2009). Another report shows in Arabidopsis shows that the defence related response was regulated by auxin signalling which was proved by *arf2* mutants (ARF2 is repressor of auxin signalling) and the regulation is independent of COI (Stotz *et al.*, 2011).

In summary, we propose a model (Figure 4.1) which compares WT and transgenic lines and observed that jasmonate insensitive phenotype in responses to salt, MeJA and IAA are mediated by in one case overexpression of OsJAZ8 and in another case OsJAZ8ΔC by which consistent and dominant suppression of jasmonate signaling is achieved. The cell elongation and increased auxin responsiveness in the transgenic BY-2 might be due to the shared components of IAA and JA signaling which would become more available for IAA signaling under suppressed jasmonate signaling leading to an increased IAA responsiveness, e.g. by increased action of AXR1. In fact, the effect was getting bigger in case of dominant repression of jasmonate signaling (Figure 3.15). This could be correlated with the results obtained in jasmonate mutant *hebiba* where absence of JA caused enhanced responsiveness to exogenous IAA by increase in coleoptile length (Riemann *et al.*, 2003, 2009).

A)



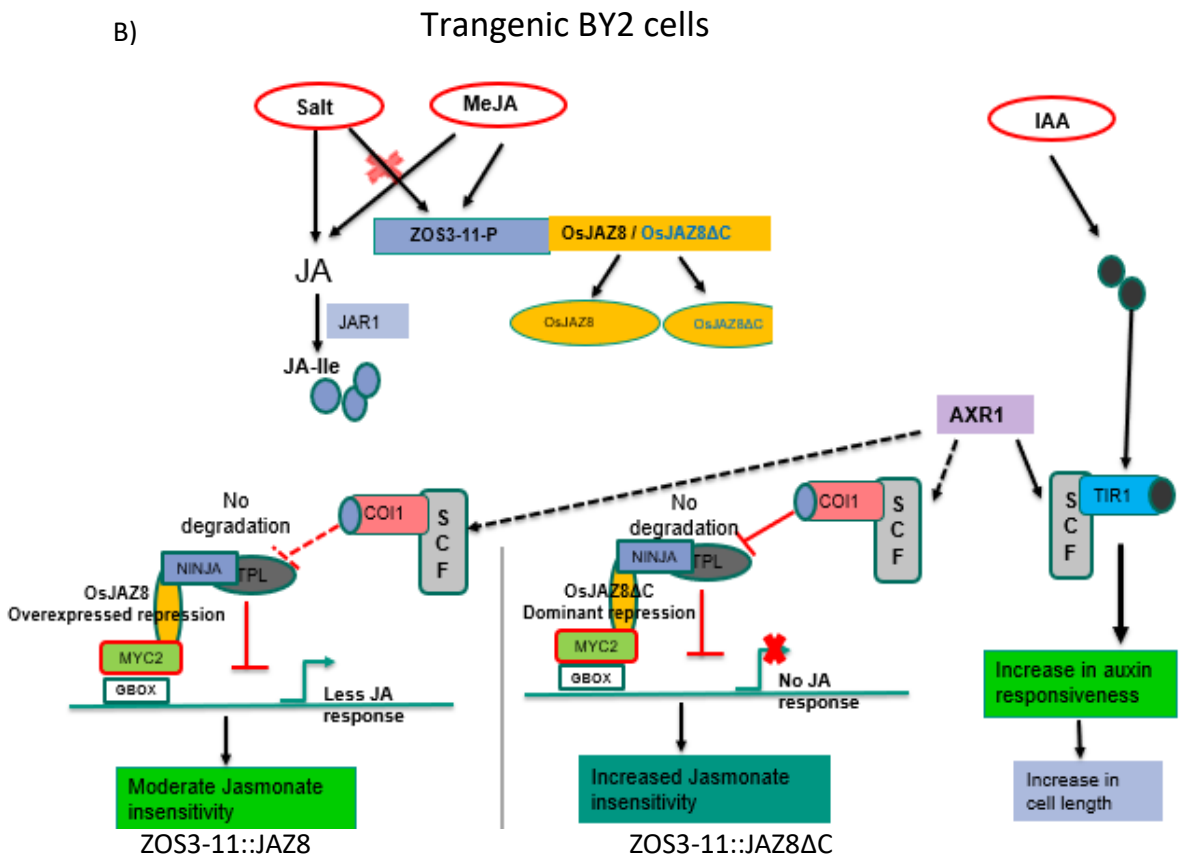


Figure 4.1: Generalized diagrammatic representation of regulation of JA signaling pathway and its cross talk with auxin signaling in (A) wild type and (B) transgenic BY-2 tobacco cells by salt, MeJA and IAA. A) In the Wild type BY-2 cells, JA-Ile produced in effect of salt and MeJA treatment activates SCF^{COI1} complex which interacts with JAZ repressor which is ubiquitinated leading to proteasomal degradation of JAZ repressor rendering jasmonate sensitive phenotype by expression of jasmonate responsive genes. Since JA signaling is active, preference of AXR1 (which mediates activation SCF complex which is shared component of both JA and IAA signaling pathway) may confer less response to IAA signaling leading to less auxin responsiveness. B) In the transgenic cell lines, salt and MeJA promote expression of *OsJAZ8* and *OsJAZ8ΔC* by activation of ZOS3-11 promoter, by which overexpressed repression by OsJAZ8 of jasmonate signaling could slow its degradation process in ZOS3-11::JAZ8 cell line, and dominant repression of jasmonate signaling by OsJAZ8ΔC blocks its degradation process in ZOS3-11::JAZ8ΔC cell line. Due to less activity of COI1, the SCF^{TIR1} is speculated to be activated by the AXR1 to respond to auxin, leading to increase in cell elongation by IAA signaling.

TPL-Topless, NINJA- Novel Interactor of JAZ (both are co-repressors), JAZ- jasmonate ZIM domain protein, TIR1-Transport Inhibitor Response 1, IAA- Indole-3-acetic acid , COI1- Coronatine-Insensitive 1, SCF- Skp, Cullin, F-box containing complex , JA-jasmonic acid, MeJA- Methyl jasmonate, JAR1- jasmonate resistant 1

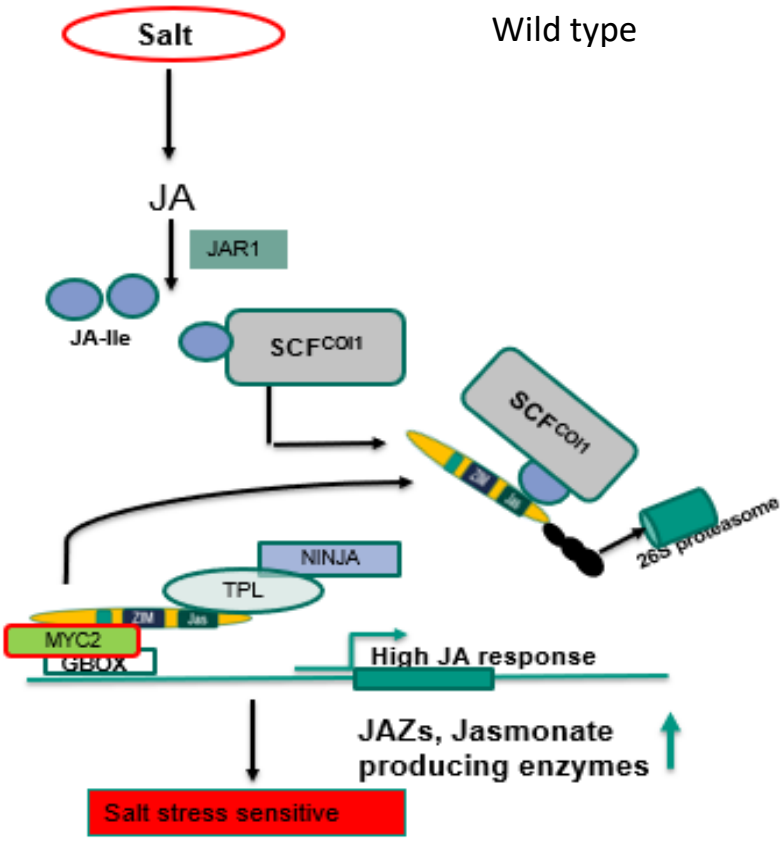
4.3 Transgenic rice shows salt stress adaptation in the early stages

Various reports demonstrate that JA-deficiency causes male sterility in case of rice (Riemann *et al.,* 2003), *Arabidopsis* (Feys *et al.,* 1994; Stintzi&Browse 2000; Mandaokar *et al.,* 2006) and other plants. In addition, expression of AtJAZ1ΔJas and AtJAZ10.4, both of which lack the Jas domain, results in male sterility (Thines *et al*., 2007; Chung&Howe, 2009). Since in our experiment, expression of *OsJAZ8* and *OsJAZ8ΔC* was under the control of salt-inducible promoters ZOS3-11 and ZOS3-12, the transgenic rice plants ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC were fertile and even showed 20% increase in the yield without salt stress (Figure 3.17).

To get more insight into the mechanism, a model has been proposed (Figure 4.2) which explains that in the transgenic rice plants ZOS3-11::JAZ8 and ZOS3-11::JAZ8ΔC, the promoter ZOS3-11 is activated by salt stress leads to overexpression of *OsJAZ8* and *OsJAZ8ΔC* in amount higher than the endogenous *OsJAZ8* in wild type. We also found higher amount of *OsJAZ8* and *OsJAZ8ΔC* even in the transgenic plants with no salt stress (Figure 3.20). This causes in case of ZOS3-11::JAZ8, suppression of jasmonate signaling due to overexpression of OsJAZ8 under salt stress. But this effect is not shown to be consistent as plants behaved similar to wild-type in the later days even though they showed better adaptability to salt stress on early state of 2nd day after salt treatment (Figure 3.18). This may be due to degradation of OsJAZ8 in the later time points, similarly to OsJAZ9 overexpressed rice reported by another group previously (Wu *et al.,* 2015). On the other hand, the ZOS3-11::JAZ8ΔC showed increased salt tolerance compared to the wild type even on the 3rd day confirms the repression jasmonate signaling by the dominant negative activity of the OsJAZ8ΔC (Yamada *et al*., 2012). Deeper investigations have to be done to know which downstream genes and pathways are regulated. Whether any difference in regulation of

antioxidants, Na^+ and K^+ uptake (Hazman *et al*., 2015; Wu *et al*., 2015) or interaction with other JAZ repression play role in adaptation to salt stress.

A)



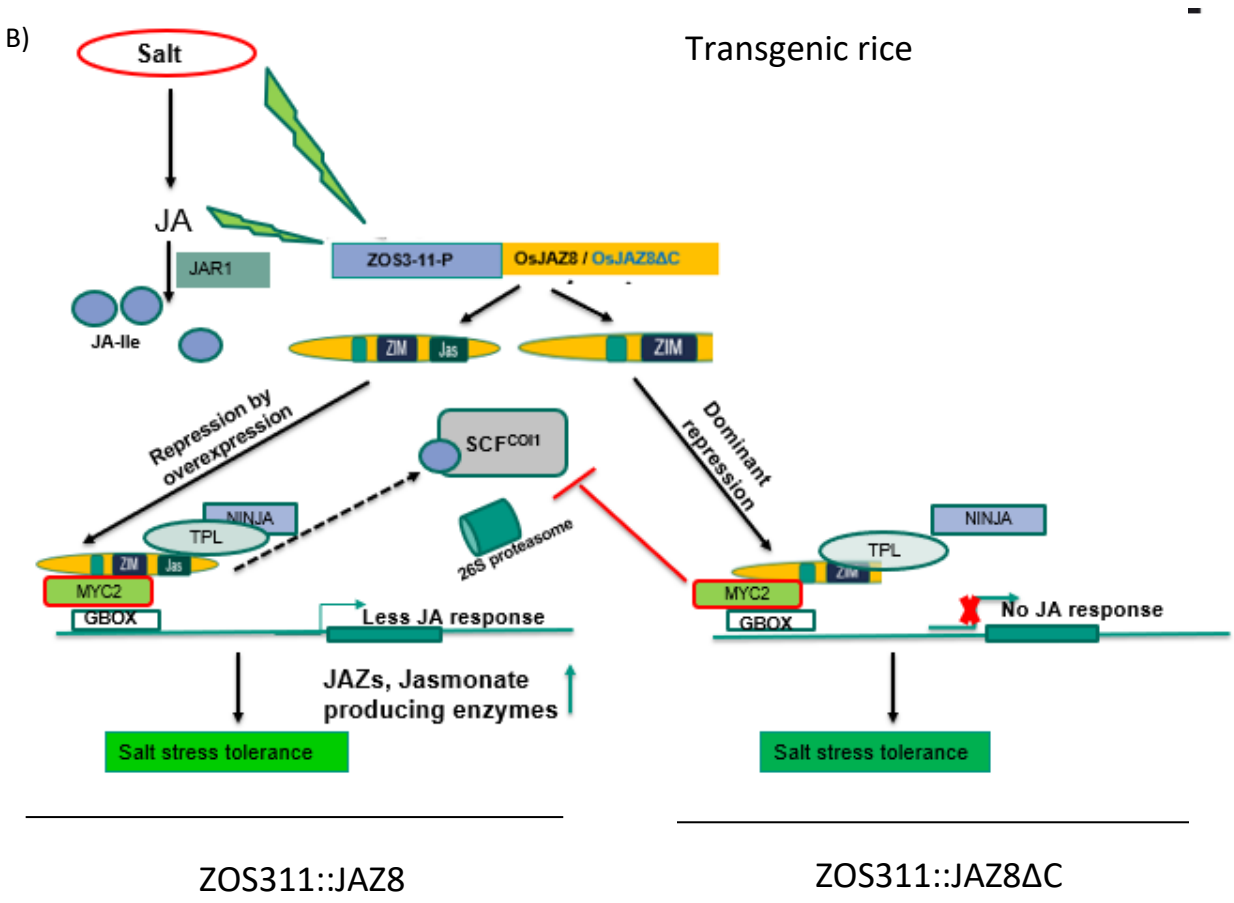


Figure 4.2: Generalized diagrammatic representation of regulation of JA signaling pathway in (A) wild type and (B) transgenic rice by 100 mM salt. A) In the Wild type rice, JA-Ile produced in effect of salt treatment activates SCF^{COI1} complex which interacts with JAZ repressor which is ubiquitinated leading to proteasomal degradation of endogenous OsJAZ8 repressor rendering jasmonate sensitive phenotype by expression of jasmonate responsive genes. B) In the transgenic rice, salt promote expression of *OsJAZ8* and *OsJAZ8ΔC* by activation of ZOS3-11 promoter, by which overexpressed repression of jasmonate signaling by OsJAZ8 could slow its degradation process in ZOS3-11::JAZ8 with decreased in expression of jasmonate responsive genes to show moderate salt tolerance (shown by light green box) , and dominant repression of jasmonate signaling by OsJAZ8ΔC blocks its degradation process in ZOS3-11::JAZ8ΔC leading to increased salt tolerance (shown by dark green box).

TPL-Topless, NINJA- Novel Interactor of JAZ, JAZ- jasmonate ZIM domain protein, COI1-Coronatine-Insensitive 1, SCF- Skp, Cullin, F-box containing complex, JA-jasmonic acid, JAR1-jasmonate resistant 1.

4.4 ZOS3-11 and ZOS3-12 are localized in nucleus and functions as transcriptional repressors

In this study, we isolated two novel genes, *ZOS3-11* and *ZOS3-12* which are coming in the family of TFIIIA known as Cys2/His2-type (C2H2) zinc finger proteins. These two genes are located adjacently on 3rd chromosome. Protein sequence analyses showed that both proteins contained two typical C2H2 ZF domains. Each ZF domain contained a QALGGH motif that is only present in plants (Figure 3.1B) and has been shown to be critical for the DNA-binding activity of plant ZPT2 proteins (Takatsuji *et al.*, 1994,1996; Yoshioka *et al.*, 2001). Both proteins lacked B-box which is a conserved motif with an amino acid sequence of KXKRSKRXR, which probably functions as a NLS showing localization in the nucleus. But experiments confirmed that ZOS3-11 and ZOS3-12 were located in the nucleus (Figure 3.2). The L-box region (EXEXXAXCLXXL), which was related to protein-protein interactions, was also found in both proteins (Figure 3.1). Furthermore, the DLN-box, which is highly conserved in both ZOS3-11 and ZOS3-12 (Figure 3.1), is typically found in ZPT2 transcriptional repressors, such as petunia PhZPT2-3 (Sugano *et al*., 2003), tobacco NtZFT1 (Uehara *et al.*, 2005), *Arabidopsis* AtAZF1, AtAZF2, AtAZF3, AtZAT7 and AtZAT10 (Sakamato *et al*., 2004; Ciftci-Yilmaz *et al*., 2007; Kodaira *et al*.,2011), and other genes. Interestingly, when expressed in yeast cells, many ZFPs with a DLN-box, including rice OsZFP179 and salt cress ThZF1 (Sun *et al*.,2010, Xu *et al.*,2007), were shown to function as transcriptional activators. We confirmed the repression activity of ZOS3-11 and ZOS3-12 using dual luciferase assay as it could repress the activity of luciferase which was induced by MeJA when under the control of OsJAZ12 promoter (Figure 3.8). This result also implies that OsJAZ12 could also be a target of these two proteins.

Phylogenetic analysis revealed that ZOS3-11 and ZOS3-12 are homologous to STZ in *Arabidopsis*. STZ was shown to be induced by salt stress and was capable of complementing yeast calcineurin null mutants and rescue its salt-sensitive phenotype (Lippuner *et al.*, 1996). Calcineurin is a Ca^{2+}/calmodulin-dependent Ser/Thr phosphoprotein phosphatase and in yeast it is an essential component in the signaling pathway through which ion channels are modulated, consequently conferring the tolerance to Na^{+} and Li^{+} ions (Nakamura *et al.*, 1993).

ZOS3-11 and ZOS3-12 were highly induced under salt stress, but the expression of ZOS3-11 was seen only in root and ZOS3-12 in both root and shoot and their induction was dependent on jasmonates (Figure 3.4) and depended slightly on ABA (Figure 3.5). This result was in correlation with STZ which exert their primary effect on the ABA-independent pathway to regulate early stress-responsive genes (Sakamato *et al.,* 2004). It was shown that STZ targets *ENA1* gene, which encodes a protein involved in the major Na^+ and Li^+ efflux system is required for manifestation of salt tolerance in yeast (Lippuner *et al*., 1996). This proves that an *ENA1*-like gene(s) may be a candidate downstream target of *AZF1, AZF3,* and *STZ* in *Arabidopsis*. In case of ZOS3-11 and ZOS3-12, more information must be gained in future to know more about its function and downstream targets.

As zinc-finger motifs of ZOS3-11 and ZOS3-12 have high structural similarity to those of the ZPT2-related proteins, they also showed DNA-binding preferences for A(C/G)T. Both proteins recognized AGT and ACT cores separated by certain number of base pair, and the best binding was found in sequence where ACT and AGT were separated by 13 bp and mutation in these core sequences causes impaired binding (Figure 3.7). Some sequence also had more than two ACT/AGT. These observations led us to speculate that each ZPT-type zinc-finger domain recognizes tandemly repeated A(G/C)T core sequences and that the spacing between each pair of A(G/C)T may be different among the ZPT2-related proteins (Takatsuji *et al.,* 1994, Sakamato *et al.,* 2004).

To find the target promoters of ZOS3-11 and ZOS3-12, further experiments have to be done like ChIP-seq to find targets promoters. Recently, a report showed that, the promoter fragment of *OsDREBIB* (DROUGHT RESPONSE ELEMENT) ranging from –1028 to –4 bp shows an enrichment in the A(G/C)T-X3-4-A(G/C)T cis-regulatory element and binding of ZOS3-12 to region between -488 bp and – 3 bp (Figueiredo *et al.,* 2012) showing the role of ZOS3-12 in abiotic stress response.

4.5 Uptake of Na^+ and jasmonate production is correlated in rice leaves

Another study as an extension of reports by Hazman *et al*., 2015, where increase in OPDA (JA precursor) was observed after 6 h of salt treatment but not in the case of JA and JA-Ile. A time

course study helped us to obtain more information in this aspect. A time course measurement of the JA, JA-Ile, its precursor OPDA and the deactivated metabolites 12OH-JA-Ile and 12COOH-JA-Ile performed in WT showed timely accumulation of all the compounds (Figure 3.21) and the deactivated products accumulation was observed to be consistent with the production of bioactive JA-Ile and also OPDA and JA showing that the deactivation process is happening simultaneously with the production of the JA and the shows an accumulative effect over time. This was not similar in case of wounding, although the degraded products accumulated over time, JA and JA-Ile showed a transient accumulation and decreased with time (Heitz *et al.*, 2012; Kurotani *et al.,* 2015). Even continuous wounding also showed transient accumulation of JA-Ile (Svyatyna *et al.,* 2014) which confirms that the production mechanism of jasmonates are different in salt stress and wounding. Deactivation of JA-Ile is Ile mediated by two Cyt P450 enzymes where it is first deactivated to 12OH-JA-Ile and subsequently to 12COOH-JA-. In Arabidopsis, CYP94B3 facilitates the initial hydroxylation step, whereas CYP94C1 preferentially catalyzes the latter reaction, although it also has an activity to hydroxylate JA-Ile (Koo *et al*., 2011; Heitz *et al.*, 2012). In rice, CYP94C2b has activities to convert JA-Ile to 12OH-JA-Ile and subsequently to 12COOH-JA-Ile (Kurotani *et al.,* 2015).

To get more insight into the sodium uptake in jasmonate mutants *cpm2* and *hebiba.* and WT, time course study was performed. Previously, (Hazman *et al*., 2015) had reported that the jasmonate mutants *cpm2* and *hebiba* showed reduced damage symptoms under salinity stress due to elevated level of antioxidants, produced longer roots and higher chlorophyll under salt stress. It was also shown that the shoots of JA biosynthesis mutants take up less Na^+-ions. However, the study was carried out in an early developmental stage and a short period of exposure to salt. It was not investigated whether different parts of rice shoots accumulate sodium ions differentially. Therefore, we carried out a salt uptake study for one week on 10 days old rice WT and jasmonate mutants *cpm2* and *hebiba.* We used a salt sensitive variety RC222 and salt tolerant variety FL478 as controls. A model showing the result is depicted in (Figure 4.3). The mutants accumulated most of the Na^+ ion and had higher Na^+/K^+ ratio in the older 4^{th} leaf and sacrificing the older leaves thus reducing the toxic effect on to the younger (5^{th} and 6^{th}) leaves and whole plant leading to adaptation to salt stress. This result was similar to the salt tolerant variety FL478. On the other hand, the WT plants were behaving similar to

the salt sensitive variety RC222 (Figure 3.22A and B) having Na^+ distributed to all the leaves making it unable to tolerate the stress. This phenomenon of sacrificing older leaf is one mechanism of the salt tolerant plants to behave during adaptation to salt stress (Wang *et al.*, 2012). To unravel more about the mechanism, we need to explore it in the future about the sodium and potassium transporters as it is known that salt tolerance is conferred by not a single factor, but is the sum of a number of contributory traits which include variation in preferential accumulation of Na^+ in the older leaves, NaCl entry via membrane leakage or apoplastic pathways using different Na^+ or K^+ channels and transporters and tolerance of NaCl within the leaf tissue with the help of various osmoprotectants. There were similar results found in other studies in rice (Wang *et al.*, 2012) where they showed mature leaves have a larger vacuole and young leaf cells less and small vacuoles and upregulation of OsNHX1, OsHKT1;1, OsHAK10, OsHAK16 expression might contribute Na^+ accumulation in old leaves of salt tolerant rice varieties, and other crop like barley (Nakamura *et al.*,1996) where accumulation of osmoprotectant glycine betaine was found in the older leaves as a result of salt accumulation. The sensitivity and high Na^+ uptake in the WT could be speculated by the action of increasing amount of JA and JA-Ile content.in the leaves with time (Figure 3.21B and C).

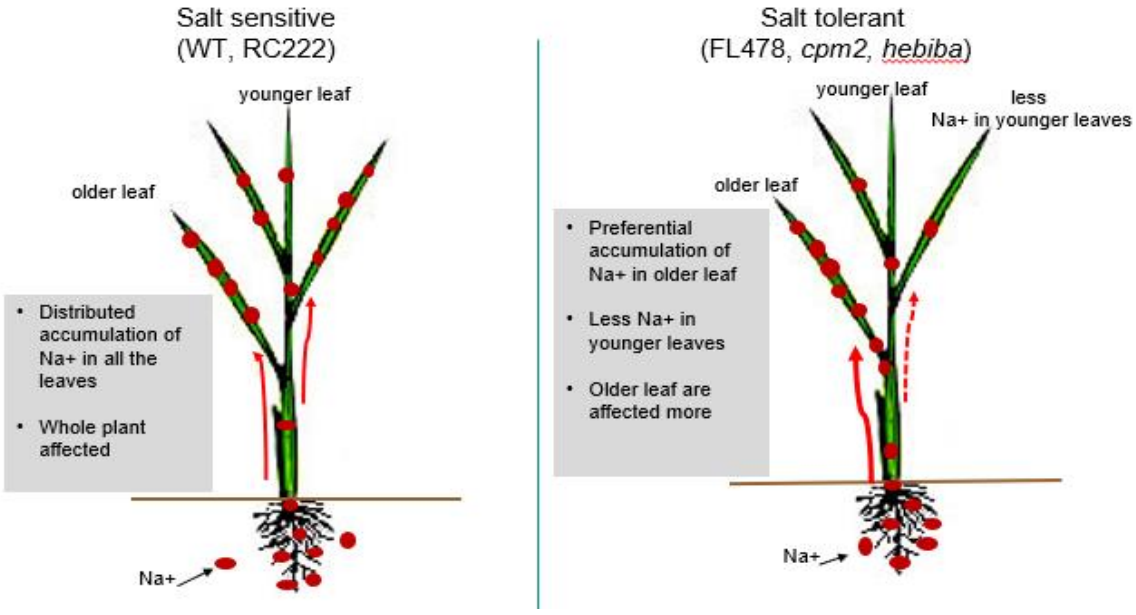


Figure 4.3: Schematic representation comparing the of Na+ ion uptake in salt sensitive 9WT, RC222) and salt tolerant (FL478, *cpm2, hebiba*).

4.6 Conclusion

The decision of the plant response to adaptation or necrosis ((which is bad for the leaf, but can be good for the plant) seems to depend on activation of OPDA signalling (necrosis) versus JA signalling (adaptation). Since plants chooses maximizing survival in the long term and farmers are interested in yield also, to have a choice of yield under stress is not natural trait. So, by modulating the temporal patterns of signalling, we might be able to manipulate this decision in favour of human selection. The novelty of this study approach was to put a signalling compound under a promoter that is activated under the stress condition and thus to shape the temporal signature of signalling.

Down regulation or absence of the phytohormone jasmonate has indicated a high risk of fertility issues and also pathogen susceptibility even though they increase the osmotic tolerance level. On the other hand, prolonged presence of jasmonate also causes programmed cell death in the plant's cells. So, it is necessary to balance the jasmonate and its signaling to increase the adaptability and fertility of the plant under salt stress condition. In the first part of this thesis, using a recombinant genetic approach to increase the jasmonate insensitivity and reduce the negative effect of jasmonate, we conclude that stability of the jasmonate repressor OsJAZ8 under salt stress plays an important role in the molecular mechanism by which JA signaling is repressed in rice plants and BY-2 tobacco cell lines. Jasmonate insensitivity was accompanied by an increase in auxin responsiveness in transgenic BY-2 cell lines. Further, the observations and experimental finding in the second part describe the characterization of two Cyc2/His2 zinc finger proteins ZOS3-11 and ZOS3-12 as a salt stress responsive transcription factors localized in nucleus binding to A(C/G)T sequence having repression activity. Further, comparing the WT and jasmonate mutants *(cpm2 and hebiba)*, the jasmonate mutants behaved similar to the salt tolerant variety showing preferential accumulation of Na^+ in the older leaves leading to salt stress tolerance, while the WT showed increased distributed accumulation of Na^+ in the leaves. This difference could be correlated with increased production of jasmonates (OPDA, JA, JA-Ile) in the WT.

4.7 Outlook

In this study, we could find some interesting results in which could be used for further improving the motive of the whole work of improving salt stress tolerance of rice. The future works extending our study could include

4.7.1 To elucidate the expression of other JAZ genes and other downstream target genes

Our results suggest that overexpression of OsJAZ8 and OsJAZ8ΔC modulate jasmonate regulatory systems leading to salt stress tolerance, but more information and clarification must be obtained in the future regarding

1. Function of other OsJAZ proteins, their binding proteins and other jasmonate dependent downstream genes.
2. Yield studies of the transgenic rice under salt stress condition.
3. Exploration and isolation of other novel and strong salt responsive genes and promoters for improved performance of our hypothesis.

4.7.2 Finding the downstream targets of ZOS3-11 and ZOS3-12

Different expression pattern of *ZOS3-11* and *ZOS3-12* in the salt tolerant jasmonate mutants and salt sensitive WT speculates important role of ZOS3-11 and ZOS3-12 in salt tolerances. Some preliminary results like obtaining cis-regulatory DNA sequence and confirmation of repression activity was done in this study, more information must be obtained to:

1. Identify the downstream targets of ZOS3-11 and ZOS3-12 to completely unravel the molecular basis of jasmonate signaling in salt stress responses using ChIP-Seq assay.
2. Produce ZOS3-11 and ZOS3-12 knockout and overexpressed rice plants, to know the exact function of these two transcription factors in the salt tolerance mechanism.

5 APPENDIX

5.1 Suspension cultures used in this study and its cultivation conditions

Name of cell line	Volume for subcultivation	Antibiotics	Sources
BY-2 Wild type	1ml	None	(Nagata *et al.*, 1992)
ZOS3-11::JAZ8	1ml	Hygromycin 40µg/ml	This work
ZOS3-11::JAZ8ΔC	1ml	Hygromycin 40µg/ml	This work
ZOS3-12::JAZ8	1ml	Hygromycin 40µg/ml	This work
ZOS3-12::JAZ8ΔC	1ml	Hygromycin 40µg/ml	This work
CONST JAZ8	1ml	Hygromycin 40µg/ml	This work
CONST JAZ8ΔC	1ml	Hygromycin 40µg/ml	This work

5.2 Coding sequences and protein sequence of the two zinc finger transcription factor

5.2.1 ZOS3-11 Locus: LOC Os03g32220, Gene model: LOC_Os03g32220.1

[Oryza sativa subsp. japonica] C2H2 zinc finger protein,

Coding sequence:
ATG GTGACCAACA TGACCCACGA TGACTACGTC TCCCTCTGCC TCATGGCGCT CGCCCAGGCG GGCGTCGGGG GTCAGTGGCC GCGCAGAAA CAGCAGATCG ACATGGCACC GCCGGCACCG GAACGCGAGC TTCTTCGGTT CCGGTGCTCC TCTGCGGCA AGGCCTTCCC GTCGCACCAA GCGCTCGGCG GGCACAAGGC CAGCCACCGC AAGCCGCCGA GGCCGCGTT GCCCATGCAC GTAATCGACG CGCCGCCGCC GCCGTCGGCC GAGGACACAG CCTCGTCGTC ACGACGACA ACAACGTCGG GCGGCGGCAG GCACAGGTGC TCGGTTTGCC ACCGTACCTT CGCGACGGGGCAGGCGCTCG GAGGGCACAA GAGGTGCCAT TACTGGGACG GGCTGTCGGT GGTGTCGGTCACGGCTTCGG CGTCAGGTTC CGGGTCGTCG AGCGTGAGGA ACTTTGACCT CAACCTGAAA CGGTGCCGG AGACGGTGGC CGCTGGCGTC AGAAGGTGGG GAGAGGAGGA GGAGGTGCAG AGCCCTTTGC CGTTCAAGAA GCGCCGGCTG CAAGCCCAT CATTGGAGCT TAATTTATAG

Amino acid sequence:

MVTNMTHDDYVSLCLMALAQAGVGGQWPAQKQQIDMAPPAPERELLRFRCSVCGKAFPSH QALGGHKASHRKPPTAALPMHVIDAPPPPSAEDTASSSTTTTTSGGGRHRCSVCHRTFATGQ ALGGHKRCHYWDGLSVVSVTASASGSGSSSVRNFDLNLKPVPETVAAGVRRWGEEEEVQSP LPFKKRRLSSPSLELNL

Note: (Yellow highlighted region is Zinc finger domain)

5.2.2 ZOS3-12: Locus: LOC_Os03g32230, Gene model: LOC_Os03g32230.1)
Coding sequence

ATGACGGCCGCCCTGCAAGCGCTGCTCGACCCGACGGCGCTGTCCTTGGGCCTGCCCAC
GCCGGCGATCAACAAGGAGGAGTACCTCGCCATCTGCCTCGCCGCGCTCGCCTGCACGC
GGGCGGGGAAGGCCCTGGTGGGAGTGGGAGGCCAGCAGCAGGTGCAGGCCTGCAATAA
GTGGCTGTGCCCAGCGCCCGCGGCGCCTGAGGAGCTCCGCTTCCGGTGCACCGTCTGCG
GGAAGGCCTTCGCCTCGTACCAGGCGCTCGGCGGACACAAGTCCAGCCACCGGAAGCCG
CCTTCCCCGGGAGACCACTACGGCGCCGCCGCCGCGGCGCAGCAGCTGGCATCCGCTGG
TGACTCGAAGGAGGACTCGGCGTCGTCAGCGGCCGGGAGCACTGGCCCGCACCGGTGCA
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TACTGGGACGGCACGTCGGTGTCCGTCTCCGTCTCGGCGTCCGCGTCGGCCGCCTCGTCG
GCCGTCAGGAACTTCGACCTCAACCTGATGCCGCTGCCGGAGAGCACAGCCGCCGCCGG
GATCAAGAGGTGGGCGGAGGAGGAGGAGGTGCAGAGTCCACTGCCGGTCAAGAAGCTC
AGGATGTCCAACTAA

Amino acid sequence:

MTAALQALLDPTALSLGLPTPAINKEEYLAICLAALACTRAGKALVGVGGQQQVQACNKWL
CPAPAAPEELRFRCTVCGKAFASYQALGGHKSSHRKPPSPGDHYGAAAAAQQLASAGDSKE
DSASSAAGSTGPHRCTICRRSFATGQALGGHKRCHYWDGTSVSVSVSASASAASSAVRNFDL
NLMPLPESTAAAGIKRWAEEEEVQSPLPVKKLRMSN
Note: (Yellow highlighted region is Zinc finger domain)

5.2.3 OsJAZ8(AK108738)

Coding sequence:

ATGGCCGGCCGTGCGACGGCGACGGCGACGGCGGCGGGGAAGGACAGGTCCAGCTTCG
CCGTCACCTGTAGCCTCCTCAGCCAGTTTCTCAAGGAGAAGAAGGGCGGCGGCGGCGGG
CTGCAGGGGCTCGGCCTCGGCTTGCGGCCGGCGCCGGCGGCGCCGCCCGCCGCGGGAGC
TGGAGGTGCTTTCCGGCCGCCGCCGACCACCATGAACTTGCTGTCGGGGCTCGACGCGCC
GGCGGTGGAGGTGGAGCCAAACACGGCGGAAACAGCGGCCGACGAGCTGCCTCTCATC
AAGGCCCCGGCCGATCAGCAAAGCGACGAAAGTGCAAGTGAGGCAGCTGGAGAGAAGG
CTCAACAGCTGACCATCTTCTACGGTGGAAAAGTAGTCGTCTTTGAGAACTTCCCGTCCA
CCAAGGTCAAGGATCTCTTACAAATCGTAAGCACAGGCGATGGCGTCGACAAGAACACC
GGCACCGCGGCAACGCAGAGCCTGCCCCGACCCGCACACAACAGTCTTCCCGATCTGCC
GATTGCAAGGAGGAACTCGCTCCATAGGTTTCTCGAGAAGAGAAAGGGCAGGATGAATG
CAAATGCTCCATACCAAGCTAACTGCACCGCTGCACCGTCCAAGCAAGCTAACGGTGAC
AAATCTTGGCTTGGGTTTGGCCAAGAGATGACAATAAAGCAGGAGATATGA

Amino acid sequence

MAGRATATATAAGKDRSSFAVTCSLLSQFLKEKKGGGGGLQGLGLGLRPAPAAPPAAGAG
GAFRPPPTTMNLLSGLDAPAVEVEPNTAETAADELPLIKAPADQQSDESASEAAGEKAQQLTI
FYGGKVVVFENFPSTKVKDLLQIVSTGDGVDKNTGTAATQSLPRPAHNSLPDLPIA
RRNSLHRFLEKRKGRMNANAPYQANCTAAPSKQANGDKSWLGFGQEMTIKQEI

5.2.4 OsJAZ8ΔC

Coding sequence:

ATGGCCGGCCGTGCGACGGCGACGGCGACGGCGGCGGGGAAGGACAGGTCCAGCTTCG
CCGTCACCTGTAGCCTCCTCAGCCAGTTTCTCAAGGAGAAGAAGGGCGGCGGCGGCGGG
CTGCAGGGGCTCGGCCTCGGCTTGCGGCCGGCGCCGGCGGCGCCGCCCGCCGCGGGAGC
TGGAGGTGCTTTCCGGCCGCCGCCGACCACCATGAACTTGCTGTCGGGGCTCGACGCGCC
GGCGGTGGAGGTGGAGCCAAACACGGCGGAAACAGCGGCCGACGAGCTGCCTCTCATC
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CCAAGGTCAAGGATCTCTTACAAATCGTAAGCACAGGCGATGGCGTCGACAAGAACACC
GGCACCGCGGCAACGCAGAGCCTGCCCCGACCCGCACACAACAGTCTTCCCGATCTG

Amino acid sequence:
MAGRATATATAAGKDRSSFAVTCSLLSQFLKEKKGGGGGLQGLGLGLRPAPAAPPAAGAG
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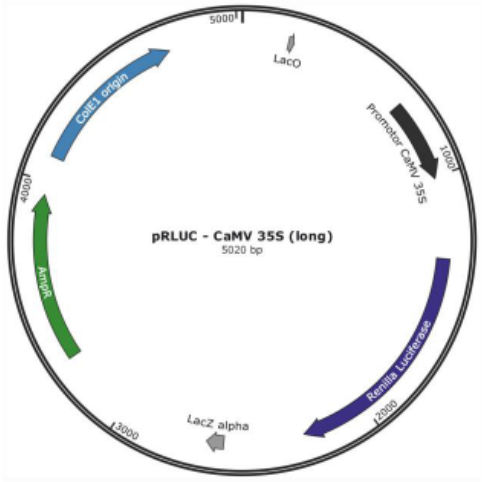
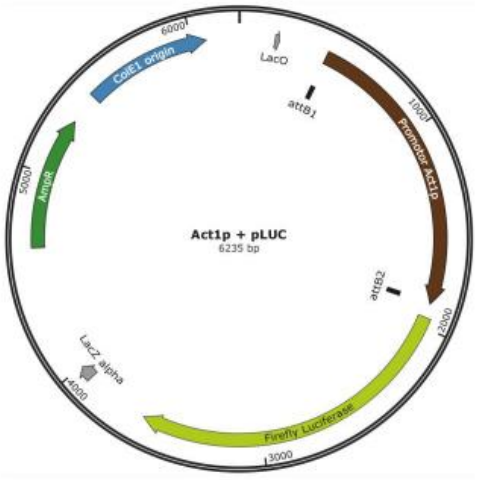
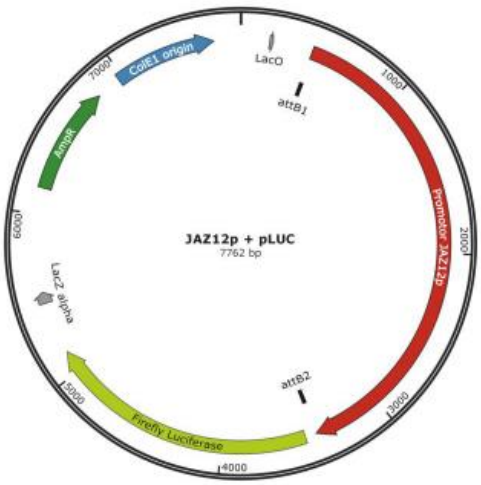
5.3 Gateway destination vectors constructed for luciferase assay



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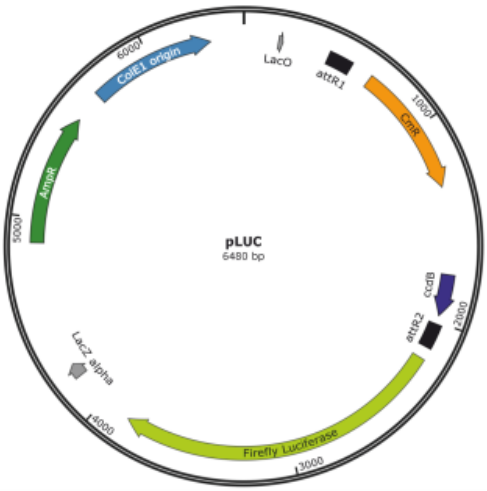
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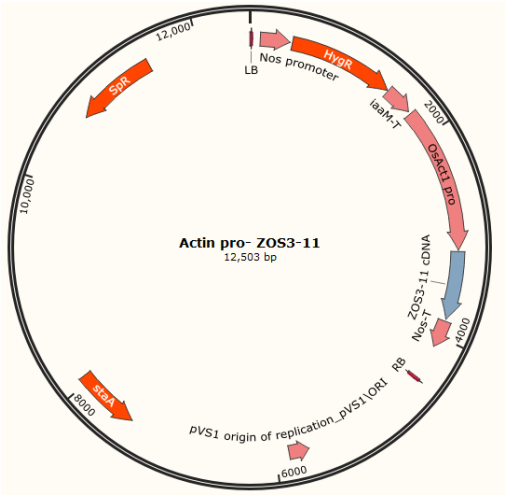
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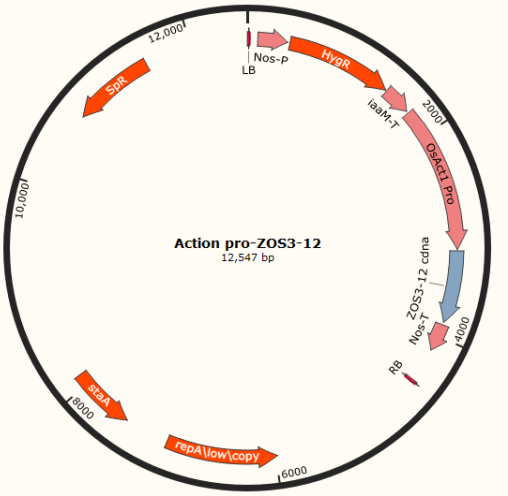
12,000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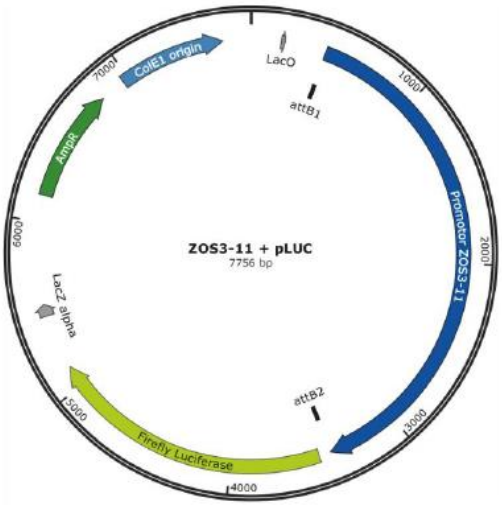
12,000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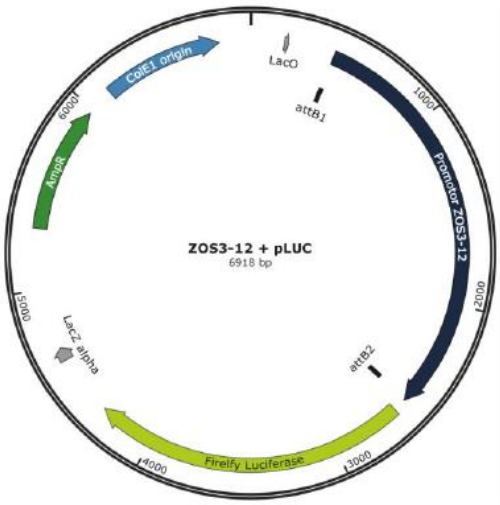
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Firefly Luciferase
LacZ alpha
AmpR
1000
2000
3000
4000
5000
6000

5.4 PCR program and components used

5.4.1 PCR for the amplification of the promoter regions.

A) The components for the 50 µl reaction mixture are listed with the volume data and the concentrations of the stock solutions.

Component	Volume
Template (gDNA 100 ng/µl)	5 µl
Q5 Reaction buffer (5X)	10 µl
dNTPs (10 mM)	1 µl
Forward Primer (20 µM)	1.25 µl
Reverse Primer (20 µM)	1.25 µl
Q5® *High-Fidelity* DNA Polymerase (2 U/µl)	0.5 µl
Nuclease-free water	31 µl

B) PCR program for the amplification of the promoter regions.

Reaction	Temp. (ºC)	Time (min:s)	Cycle
Denaturation	98	0:30	1
Denaturation	98	0:10	
Annealing	60-68[1]	0.30	35
Elongation	72	1:45[2]	
End elongation	72	2:00	1
End	10	∞	1

1 Annealing temperature with NEB TM Calculator of New England Biolabs (Frankfurt am Main)

2 Elongation time for 3 kb

5.4.2 PCR approach for gene expression analysis.

A) The individual PCR components with their concentrations and the volume data for a 20 µl reaction mixture are listed

Component	Volume
Template (cDNA 1:50)	5 µl
Taq Reaction buffer (10X)	2 µl
dNTPs (10 mM)	0.4 µl

Forward Primer (20 µM)	0.4 µl
Reverse Primer (20 µM)	0.4 µl
Taq DNA Polymerase (5 U/µl)	0.08 µl
Nuclease-free water	11.7 µl

B) PCR program for gene expression analysis.

Reaction	Temp. (ºC)	Time (min:s)	Cycle
Denaturation	94	0:30	1
Denaturation	94	0:10	
Annealing	60[1]	0.30	35
Elongation	68	1:45[2]	
Endelongation	68	2:00	1
End	12	∞	1

1 Annealing temperature with NEB TM Calculator of New England Biolabs (Frankfurt am Main)

2 Elongation time for 3 kb

5.4.3 qPCR approach

A) Indicated are the individual components with volume data for a 20 µl batch. In addition, the concentrations of the starting solution are listed.

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

B) qPCR program.

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

5.5 Reaction step and incubation steps of cDNA synthesis

Component	Volume
Template (total RNA)	1 µg
Oligo-dT Primer (40 µM)	2 µl
dNTPs(10mM) ,	1 µl
Nuclease free water	upto 16 µl
Incubation: 5 Minute 70°C, briefly centrifuge, place on ice	
Reverse transcriptase buffer (10x)	2 µl
RNase Inhibitor (10U/µl)	1 µl
M-MuLV Reverse Transcriptase (200 U/µl)	1 µl
Incubation: 1 hour at 42°C, 10 minutes at 90°C, storage: -20°C	

5.6 List of cloning and sequencing primers.

5.6.1 PCR primers with the Gateway® attB sequences for the amplification of the promoter regions and coding sequences of ZOS3-11 and ZOS3-12 and primers with restriction site (underlined) for amplifying coding sequence of OsJAZ8 and OsJAZ8ΔC

Primer	Forward Primer	Reverse Primer
ZOS3-11 promoter	GGGGACAAGTTTGTACAAAAAAGCA GGCTTGGAACTTCTAAGTGGTTGTGC CTTA	GGGGACCACTTTGTACAAGAAAGCTG GGTCTTTTGTATGATGCGCGTGAACTG
ZOS3-12 promoter	GGGGACAAGTTTGTACAAAAAAGCA GGCT GTGACCCAAGCTGATGCAC	GGGGACCACTTTGTACAAGAAAGCT GGGTCCGAGGTACTCCTCCTTGTTGAT
ZOS3-11 cDNA	GGGGACAAGTTTGTACAAAAAAGCA GGCTC ATGGTGACCAACATGACCC	GGGGACCACTTTGTACAAGAAAGCTG GGTC TAAATTAAGCTCCAATGATGGG
ZOS3-12 cDNA	GGGGACAAGTTTGTACAAAAAAGCA GGCTC ATGACGGCCGCCCTGCAAGC	GGGGACCACTTTGTACAAGAAAGCTG GGTC GTTGGACATCCTGAGCTTCTT
OsJAZ8	GGCGCGCCAGTGGTGGTGGTGGTGG TGAG	GGGCCCGAGCTCCCCCACAGATCACT TGACAC
OsJAZ8ΔC	GGCGCGCCAGTGGTGGTGGTGGTGG TGAG	GGGCCCGAGCTCCAGATCGGGAAGAC TGTTGTG

5.6.2 The primers for sequencing and checking the inserts (promoter regions ZOS3-11 and ZOS3-12) and *OsJAZ8* and *OsJAZ8ΔC* The primer labels and their sequences are listed in the 5'-3 'direction

Primer name	Forward Primer	Reverse Primer	Tm temp	Product length
ZOS3-11 promoter	ZOS3-11-jas8 FP1 CCGAGCTCGAATTATCACAA	ZOS3-11-jas8 RP1 CGACGCATATTTTGCTTTCA	53	814
ZOS3-11 Promoter	ZOS3-11-jas8 FP2 CAGTGGGATCCACATGTCAG	ZOS3-11-jas8 RP2 TTGGAGCGATCCATAAAGAGA	55	796
ZOS3-12 promoter	ZOS3-12-jas8 FP1 GTTTTCCCAGTCACGACGTT	ZOS3-12-jas8 RP1 TTGCTTTATTAGGCGCAGGT	57	691
ZOS3-12 promoter	ZOS3-12-jas8 FP2 TTTGTGAATTCCCTCCATCC	ZOS3-12-jas8 RP2 GTGTGGGCTGGCTTTTAGTC	55	684
DEST RP	JAS8 FP2 AATCGTAAGCACAGGCGATG	DEST RP TTGCGGGACTCTAATCATAAAAA	53	497
NtGADPH	ACAAATTGCCTTGCTCCCTTGGC	CCTCCAGTCCTTGGCTGATGG	63	

5.6.3 Primers used for qPCR. The primer sequence along with their names are indicated

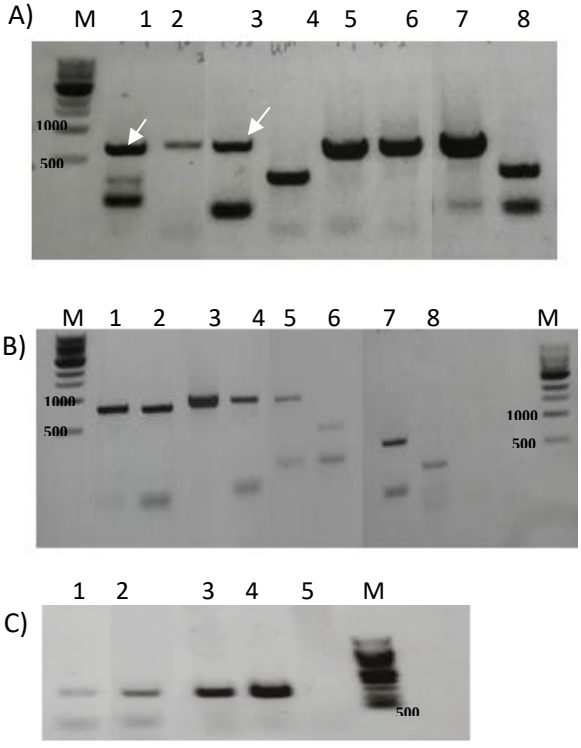
Primer	Forward Primer	Reverse Primer	Product length
OsJAZ8	JAS81 FP ACGAAAGTGCAAGTGAGGCA	JAS81 RP GGTGGACGGGAAGTTCTCAA	95
NtGADPH NM_001325431	ACAAATTGCCTTGCTCCCTTGGC	CCTCCAGTCCTTGGCTGATGG	132

OsZOS3-11	ZOS3-11Q.F3 GTCAGGTTCCGGGTCGTC	ZOS3-11Q.R2 CTCCTCCTCTCCCCACCTT	105
OsZOS3-12	ZOS3-12Q.F GATCTGCCCATTGATCGACT	ZOS3-12Q.R CCTTGGCGACAATCAATCTT	149
UB5	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT	69

5.7 SDS PAGE -Gel casting reagents

Component	Separation Gel	Stacking Gel
Acrylamide (30 % [v/v] acrylamid 0.8 % [v/v] bisacrylamid)	8.2 ml	1.3 ml
Separation buffer (1.5 M Tris-HCl, pH 8.8)	6.2 ml	
Stacking buffer (0.5 M Tris-HCl, pH 6.8)		2.3 ml
H2O	10.3 ml	6.2 ml
10 % [w/v] APS	215.9 µl	105 µl
40% TEMED	108 µl	52.8µl

5.8 Checking T-DNA inserts using PCR



Confirmation of T-DNA inserts in BY-2 transgenic cell lines by PCR. The inserts have been checked in parts. ZOS3-11 and ZOS3-12 promoter region checked with two different pairs of primers. **A)** constructs with promoter ZOS3-12 depicted in lane 1,5 (size 691) and 2,6 (684 bp); lane 3, 7 for Hygromycin; lane 4,8 for GADPH (140bp). (white arrow shows the correct size). **B)** Constructs with promoter ZOS3-11-depicted in lane 1,3 (size 814 bp) and 2,4 (size 796 bp) for promoter ZOS3-11; lane 5, 7(size 750bp) for Hygromycin; lane 6, 8 (132 bp) for GADPH. The right part (lane 7, 8, M) of the gel has been taken from another gel picture. **C)** Confirmation of OsJAZ8 (size 490bp) and OsJAZ8ΔC (size 300b) inserts, lane 1-ZOS3-12::JAZ8 , lane 2- ZOS3-12::JAZΔ8, lane 3- ZOS3-11::JAZ8, lane 4-ZOS3-11::JAZΔ8 and lane 5-WT

5.9 Transgenic rice lines used for this study

Zos3-11::JAZ8	**Zos3-11::JAZ8ΔC**	**Zos3-12:: JAZ8ΔC**
MR290 19.5 (1)	MR291 4.1	MR 293 20.1(1)
MR290 10.4(2)	MR291 4.1(2)	MR292 2.1(2)
MR290 12.2(2)	MR 291 4.1(2)	MR 293 13.1(1)

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