Study of WRKY Overexpression in Tomato

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Minor Thesis Report

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INTRODUCTION

1.1. Tomato (Solanum lycopersicum L.)

Tomato is one of the most important vegetables in the world. It is consumed as a fruit and vegetable but its high consumption is also due to processed products such as purée, juice, ketchup and sauce (Gould, 1992). In addition, the major carotenoid in tomato, lycopene, is one of the most potent antioxidants among dietary carotenoids and is believed to have a beneficial effect on human health (Agarwal & Rao, 2000). Therefore, the tomato is emerging from a dietary product to a medicinal and cosmetic product.

Thousands of tomato cultivars have been generated through breeding. Tomato breeding has been extremely successful in creating new varieties suitable for different environments and also with different fruit types and sizes suitable for consumption in different cultures and regions. The total production of tomatoes in the world in 2011 was 159.347.031 tonnes (FAOSTAT, Database/ http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/QC/).

Tomato production is often hindered by abiotic and biotic stresses. Most research has focused primarily on the damage caused by biotic stress, but problems from abiotic stress are also increasing. One major abiotic stress is salinity stress. Plant growth and development are highly affected by salinity stress. More than 25% of the irrigated land world-wide (about 60 million hectares) is experiencing increased salinity (Ghassemi F, 1995). Improvement in tomato production will require the development of several steps aimed at enhancing the tolerance of tomatoes to saline soils. This will necessitate a greater understanding of the genetic, physiological, biochemical and molecular mechanisms that underlie salinity stress responses as well as the pathways that lead to salinity tolerance, in addition to improvements in cultivation techniques and breeding programs.

1.2. Mechanisms of Salt Tolerance in Plants

Salinity stress involves interconnected biochemical and molecular pathways. In plant, salinity causes stress via osmotic stress and ion toxicity. Osmotic stress occurs due to shortage in the availability of water. In later stage (usually after two weeks), ion toxicity occurs. Zhu, Hasegawa, Bressan, & Bohnert, (1997) stated that the molecular regulation of salt tolerance is complex and involves the production of stress proteins and other compatible osmolyte compounds. Abiotic stress resistance mechanisms are known to be interconnected. Reactive oxygen species (ROS) is a product of aerobic metabolism. ROS can be destructive or can help plant as second messenger in several cellular processes (Yan, Tsuichihara, Etoh, & Iwai, 2007). Key points in the existence of ROS are the concentration and the balancing between productions and scavenging. When the level of ROS exceeds tolerance level, it causes ion toxicity, osmotic stress and cellular damage in plant cell. In plant ROS is produced in chloroplast, mitochondria, peroxisomes, plasma membranes, reticulum endoplasmic, cell walls and apoplast (Heyno, Mary, Schopfer, & Krieger-Liszkay, 2011).

The mechanism underlying tolerance to salinity stress is complicated, as it involves: (1) compartementiation or exclusion of ions; (2) ion uptake controlled by the transport of ions from roots to leaves; (3) production of compatible solutes; (4) changes in photosynthesis; (5) modification of membrane structure; (6) induction of antioxidative enzymes; and (7) induction of hormonal signalling pathways (Parida and Das, 2005). Vinocur and Altman (2005) proposed a model that interconnected the molecular and biochemical pathways of abiotic stress tolerance responses. Salt stress caused cellular damage and even resulted in oxidative stress. Salinity activated the synthesis and accumulation of ABA in rots and shoots (J. Zhang, Jia, Yang, & Ismail, 2006). The cellular adaptation pathway involves the perception of a signalling stress by signal sensing and transduction through proteins such as osmosensors (e.g. AtHK1), phospholipid-cleaving enzymes (e.g. PLD), second messengers (e.g. Ca^{2+}, PtdOH, ROS), MAP kinases, Ca^{2+} sensors (e.g. SOS3), calcium-dependent protein kinases (e.g. CDPKs). The signal induces transcription factors (such as CBF/DREB, ABF, HSF, bZIP,

MYC/MYB and WRKY) which transfer the signal to genes and activate the stress response mechanisms responsible for detoxification or ROS scavenging (SOD, APX), chaperone functions (Hsp, SP1, LEA or COR), synthesis of osmoprotectants (e.g. proline, glycine betaine (GlyBet), polyols) and ion movement (e.g. aquaporins and ion transporters). This re-establishes cellular homeostasis, functional and structural of protection of proteins and membranes allow plant to have salt tolerance or resistance (Vinocur & Altman, 2005).

1.3. Biotechnology in Breeding for Tomato Salt Tolerance

Improvements in salt tolerance in plant can be established using two approaches: by conventional breeding or by biotechnology. The conventional approaches improve salt tolerance in elite genotypes using wild species as donors of salt tolerance traits. This approach has limitations especially in the selection stage, which requires considerable time. Environmental effects can also influence field selection and can limit the stability of the phenotypic (Cuartero, Bolarin, Asins, & Moreno, 2006). For these reasons, biotechnological approaches can resolve these limitations.

Two main approaches are taken when using biotechnology in breeding to improve salt tolerance. First is an approach involving molecular markers for the mapping of quantitative trait loci (QTLs), followed by marker-assisted selection. The second approach is to use transformation technology for the introduction and expression of novel genes involved in salt tolerance obtained from other organisms (Yamaguchi & Blumwald, 2005); (Cuartero et al., 2006).

The advances in breeding programs using biotechnology allow many approaches to improve salinity stress tolerance, which is controlled by complex traits. The use of QTL analysis allows determination of the putative contribution of genes (Cuartero et al. 2006). The evaluation of F2 populations from crosses between *Solanum lycopersicum* and two wild relatives (two accession from *S. pimpinellifolium, S. galapagense* and one accession from *S.galapagense* S.Darwin&Peralta) indicate that 43% of the loci were linked to QTLs for salinity tolerance (Monforte, Asíns, & Carbonell, 1997a). Unfortunately, the tomato

QTLs for salt tolerance vary in response to different environments (e.g. saline or non-saline conditions, or different degrees of salinity) (Monforte, Asíns, & Carbonell, 1997b). The presence of this epistasis makes MAS (Marker Assisted Selection) inconvenient because QTL effect may be environmentally sensitive (Gurganus et al., 1998). Cuartero et al., (2006) suggested that the use of RILs (Recombinant Inbreed Lines) or a DH (Double Haploid) population can be the solution for multi-trait analysis and the study of epistasis interaction with respect to salt tolerance.

The study of genes involved in salt tolerance began with the overexpression of the yeast (*Saccharomyces cerevisiae*) gene, *HAL1*. Overexpression of this gene can improve salt tolerance in yeast by regulating the K^+/Na^+ concentration during salt stress (Serrano & Gaxiola, 1994); (Serrano, 1996). Gisbert et al., (2000) introduced the *HAL1* gene into tomato (*Lycopersicon esculentum* Mill.) by *Agrobacterium tumefaciens*-mediated transformation and showed that the progeny of transgenic plants had a higher salt tolerance compared to untransformed control plants. Further research determined that the *AtNHX1*gene from *Arabidopsis thaliana* is involved in vacuolar Na^+/H^+ antiport (Apse, Aharon, Snedden, & Blumwald, 1999). H.-X. Zhang & Blumwald, (2001) were succeeded in overexpressing this gene in tomato and showed that transformed tomato plants were able to produce fruits in the presence of 200 mM NaCl and that high accumulation of NaCl occurred in the leaves but not in the fruit. However, after several selections in different regions, the transgenic *AtNHX1*gene plants failed to show higher tolerance when compared to control plants (Flowers, 2004).

Another biotechnological approach for increasing the success rate when breeding for salt tolerance is by utilizing plant transcription factors (TFs). Plant transcription factor genes have a role in regulating the network that controls plant tolerance or resistance mechanisms (W. J. Chen & Zhu, 2004; Huang et al., 2012). The genome-wide analysis of the WRKY TFs in tomato has been published, and shows 81 SlWRKY genes that are classified into three main groups. One of these genes, SlWRKY80, has a positive effect on salt and drought tolerance in tomato (Huang et al., 2012). Further information about

WRKY TFs, their function in defense mechanisms, and future prospects in breeding for salt tolerance will be described in the next section.

1.3. WRKY Transcription Factors

WRKY transcription factors are one of the ten largest transcription factor families across the green lineage and are involved in signalling webs that regulate important plant processes (Rushton, Somssich, Ringler, & Shen, 2010). Reports have been published on genome identification and mapping of WRKY in eukaryotes. In 2000, T Eulgem, Rushton, Robatzek, & Somssich, (2000) identified more than 70 WRKY genes in Arabidopsis. A further investigation on the evolution of the WRKY transcription factors by Y. Zhang & Wang, (2005) indicated that a single copy of the WRKY gene, which encodes two WRKY domains, was found in a primitive eukaryote, *Giardia lamblia*; a slime mould closely related to the evolutionary lineage of animals and fungi, in *Dictyostelium discoideum*, and in *Chlamydomonas reinhardtii*, a green algae that represents an early evolutionary branch of plants. Other studies have investigated WRKY genes in plant genomes and have reported 45 WRKYs in barley (Mangelsen et al., 2008), 46 WRKYs in canola (Yang, Jiang, Rahman, Deyholos, & Kav, 2009), 64 WRKYs in soybean (Q. Zhou et al., 2008), 83 WRKYs in pine (*Pinus monticola*) (Liu & Ekramoddoullah, 2009) and 102 WRKY in rice (*Oryza sativa)* (Wu, Guo, Wang, & Li, 2005). Most recently, 136 WRKY proteins coded by 119 genes were reported in maize (Wei, Chen, Chen, Wu, & Xie, 2012) and 58 WRKY genes were found in the physic nut (*Jatropha curcas* L.) (Xiong et al., 2013).

WRKY transcription factors are involved in plant growth regulation and growth and development (Mao et al., 2011; Rushton et al., 2010; Ulker & Somssich, 2004). WRKY members play a pivotal role in trichome morphogenesis (Taji et al. 2002), cell maturation (Birnbaum et al., 2003) and gibberellin signalling pathways (Z. Zhang et al., 2004). WRKY members also regulate seed development, seed dormancy and germination, root formation, senescence, metabolic pathways and responses to abiotic and biotic stress (T. Eulgem & Somssich, 2007; Pandey & Somssich, 2009; Rushton et al., 2010).

1.5. *WRKY Overexpression in Salt Stress*

Most of gene expression studies are using overexpression of target genes. Overexpression of genes has been used to uncover the gene expression, unraveling gene systematic and genome-wide analysis of gene function. Overexpression of transcription factors is a great tool to understand the role of TFs in plant development and stress responses. Besides, more phenotypes and unexpected phenotypes can be generated by overexpression that are less affected by redundancy (compared to knock out and knock down), and in most cases the gene functions are revealed by overexpression (J. Z. Zhang, 2003).

Overexpression of WRKY TFs have been used to reveal the function of this gene family in abiotic stress especially in salt stress. (Qiu & Yu, 2009) reported that overexpression study of *OsWRKY45* in Arabidopsis improves salt and drought tolerance and *OsWRKY30* is activated by MAP kinases to confer drought tolerance in rice (Shen et al., 2012). Moreover, overexpression of WRKY25 and WRKY33 in Arabidopsis can induce higher salt tolerance (S. Li, Fu, Chen, Huang, & Yu, 2011) while overexpression of AtWRKY30 resulted salt tolerance during germination stage (Scarpeci, Zanor, Mueller-Roeber, & Valle, 2013). Overexpression of soybean GmWRKY54 in Arabidopsis elevated the expression of stress responsive genes of DREB2A and STZ, and enhanced Arabidopsis tolerance to salt and drought (Q. Zhou et al., 2008). Likewise, ooverexpression of maize ZmWRKY33 in Arabidopsis activated stress-induced genes under normal treatment and enhanced salt stress tolerance under stress treatment (H. Li et al., 2013). The ABA signalling has an effect on the AtWRKY60 activation in salt tolerance mechanism (H. Chen, Z. Lai, J. Shi, Y. Xiao, Z. Chen, X. Xu, 2010). Furthermore, overexpressing a wheat TaWRKY19 improved salt tolerance, drought and freeze stresses by elevating the expression of stress responsive genes of DREB2A, RD29A, RD29B and Cor6.6 (Niu et al., 2012). Similarity, TaWRKY10 has a function in regulating osmotic balance, ROS scavenging and transcription of stress related genes under drought and salt stresses (C. Wang et al., 2013). *Brasica campestris* spp. *Chinensis* BcWRKY46 expression in tobacco

reduced the susceptibility of transgenic tobacco to freezing, ABA, salt and dehydration stresses (F. Wang et al., 2012). In *Tamarix hispida*, transformation of overexpression ThWRKY4 in arabidopsis showed the improving activities of superoxide dismutase and peroxidase, decreasing levels of O_2^- and H_2O_2, reducing electrolyte leakage, keeping the loss of chlorophyll, and protecting cells from death (Zheng et al., 2013). A novel WRKY gene, DgWRKY3, from chrysanthemum (*Dendranthema grandiflorum)* was identified. The DgWRKY3-overexpression tobacco plants is upregulated by salinity stress which increase salt tolerance. The osmotic adjustment was resulted by the increased levels of proline, reduced accumulation of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), higher activities of antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) and the greater accumulation of antioxidants including ascorbate (AsA) and glutathione (GSH) (Q.-L. Liu et al., 2013). Taken together, overexpression mechanism in analysis of genes is emerging as the effective strategy in finding out WRKY gene expression under specific condition. And WRKY TFs are arise as as key regulators in salt stress tolerance responce regulating hormones (ABA) signalling, proteins, osmoprotectors, antioxidants, and other transcription factors. The exploration of tomato's WRKY still very limited especially related to salt tolerance. Therefore aims of this study are: 1) to validate and verify WRKY transcription factor overexpression in tomato transgenic lines 2) to examine the effects of different tomato WRKY genes at the gross phenotypic level 3) to examine their involvement in salt stress tolerance by examining morphological, physiological, biochemical and molecular characterisation of the overexpression lines in the presence of salt stress and 4) to identify WRKYs that can contribute for the improvement of the resistance tomato to salt stress without unintended pleiotropic effects and the underlying mechanisms underlying it.

METHODOLOGY

2.1. Plant Materials

The seed of overexpression WRKY tomato lines (T1) and cv. money maker (Table 1) were disinfected as follows : i) Seeds were washed in sterile water in ethanol for one minute, ii) The seeds were then sterilized in 1.5% NaOCl solution for 15 minutes followed by three washes in sterile water. iii) The disinfected seeds were then sown in MS medium (pH 5.8, 0.8% agar) supplemented with the antibiotic kanamycin 100 mg/l) for the selection of plants carrying the transgene (except for MM). After 3-4 weeks, plantlets were propagated on MS medium. The rooted shoots were used in the green house experiment.

2.2 . Salt stress Application

The rooted shoot tip *in vitro* plantlets of overexpression WRKY and MM were acclimatized in vermiculate medium (3L pot). For the first three days, plants were covered by plastic cup to reduce transpiration. Plants were watered with ½ Hoagland medium. After two weeks in green house, the plants are treated with 0 mM and 100 mM NaCL for 4 weeks. Each line had 4 replications spread to 4 different blocks.

2.3. Traits and Measurements

2.3.1. Morphology

Shoot length of the plants was measured at 4 weeks after application of salt stress and harvesting time. The fresh weigh of leaves and stems ware determined at the harvesting day. To obtain dry weight, leaves and stems were dried in 70°C for 72 hours. The Relative fresh weight and relative dry weigh from plant under salt treatment were calculated against the leaves and stems fresh and dry weight at control condition.

Table 1. Genotype used in thesis experiment

Unigene in Tomato	CODES	SGN	P-Blast Arabidopsis	Description
SGN-U565155	WRKY1-1 WRKY1-2 WRKY1-3	WRKY8	15,11,17,39,74	As WRKY 15: many interactions, including ERF1, WRKY 33, HSFs etc. Moderately expressed, induced drought/salt/ pathogen
SGN-U565159	WRKY2-2	no hit	27,29,65,16, 22	As WRKY29: interactions with mpk3,6
SGN-U571282	WRKY3-1 WRKY3-2 WRKY3-3	WRKY6 induced under drought	42,6,31,72	As WRKY6: interactions with ZAT6,WRKY33, RLK
SGN-U587314	WRKY4-1 WRKY4-2 WRKY4-3	WRKY22 repressed under drought/potentially increased under biotic	27,22,29,65, 16	As WRKY22: interactions with MPK3, oxidative stress protein
SGN-U602602	WRKY5-1	no hit		
SGN-U563809	WRKY7-1 WRKY7-3	WRKY11 highly expressed, induced salt/drought, mixed response to pathogens	11,17,39,74	As WRKY 11: interactions with CAMs, WRKY33
SGN-U576890	WRKY8-1	WRKY10 low expressed, induced by pathogens	11,74,15,17,19	As WRKY 74: interactions with CAMs, RLKs for resistance to pathogens
SGN-U577936	WRKY9-2 WRKY9-3	WRKY48 slightly induced by drought/pathogen	71,28,57,68, 43	AS WRKY 71 interactions with DREB, ERF etc. AS WRKY 28: interactions with ICS, MYB

2.3.2. Physiology

Chlorophyll content was measured by using SPAD meter. The measurement was taken in sixth or seventh leaf from the top of the plant. The chlorophyll content was calculated as the average of these two measurements.

Electrolyte leakage was determined by cutting fresh leaf disks with a cork borer (~7mm diameter) of each genotype on control condition (0 mM NaCl). 12 leaf disks were placed on the 50 ml plastic tube containing 20 ml of MQ water with 0.5 and 1 µM paraquat then placed under 24 hours light at room temperature. The electrical

conductivity (ECi) was measured using an EC meter after 24 hours, 48 hours and 72 hours. To get final electrolyte leakage (ECf), the leaves were autoclaved at 121°C, 15 psi for 5 minutes and cooled to 25°C. The electrolyte leakage (EL) was counted following (Dionisio-Sese & Tobita, 1998) formulation : EL = ECi/ECf×100.

2.3.3. Biochemical

Ion content analysis was carried out using ion chromatography.

2.3.3.4.Materials grinding and Ashing

Dry leaves and stems were ground separately using grinding machine with 1 mm^2 mess. For the ashing preparation, leaves and stems were weighted. The weight of each sample was 29-30 mg. For ashing, materials were placed in an oven for at least 6 hours at 580°C.

2.3.3.5.Analytes Preparation

After cooling down, formic acid (3M) was added to each ashed sample followed by shaking for 15 minutes at 99°C and cooled to room temperature. Afterwards, 9 ml of miliQ (pure water) was added to the sample. Then, 200 µl of the sample was added to 9.8 ml of miliQ (500x dilution) in special IC analysis plastic tube. Then those samples were ready for ion chromatography analysis according to standard procedures provided by the manufacturer.

2.3.3.6.IC content analysis

The ions Cl^-, PO_4^{3+}, SO_4^-, Na^+, K^+, Mg^{2+} and Ca^+ of leaf and stem were analyzed using IC equipment of Metrohm. Anion measurement was by Metrohm 881 Compact IC pro (2.882.0020) using Metrosap A 150, 150/4.0 mm column equipped with a Metrosep C5/5 Supp 4/6 Guard column. Cation measurement was analyzed by Metrohm 881 Compact IC pro (2.882.0010) using Metrosap C4 Supp 4, 250/4.0 mm column equipped with a Metrosap A Supp 4/6 Guard column.

2.5. Analysis Transgenic WRKY Overexpression Lines of Tomato by Molecular Identification

2.5.1. Sample collection

Gene expression analyses were carried from leaflets three weeks olds, the second leaf counting from top of all genotypes (transgenic and WT) in control (0 mM salt treatment) and salt treatment. Afterwards, leaves were ground with liquid nitrogen. About 100mg of ground sample was used for RNA isolation.

2.4.2. RNA isolation

RNA extracted from grinded samples using RNeasy mini kit (Qiagen). The quality and purity of RNA was checked using 2% agarose gel. The quantity of RNA was checked by a Nanodrop device.

2.4.3.4. RNA purification

One microgram of RNA was mixed with miliQ water (8 µl) and 2 µl of buffer (consisted of :1 µl Dnase reaction buffer, 1 µl Dnase, 1 µl amp grade , Invitrogen). Each tube contained 10 uL (RNA, water and buffer). The tubes was treated at room temperature for 15 minutes to allow digestion of the volunteer DNA. Afterwards to inactivated DNAse, 1 µl EDTA was added and the samples were incubated at 65°C for 10min.

2.4.3.5. *Reverse Transcrption*

The Reverse Transcription reaction mix (20 µl) consisted: 5x iscript reaction mix (4 µl), iscript reverse transcriptase (1 µl) (Iscript™ cDNA Synthesys Kit, BioRAD), RNA template (11 µl) and water for the rest of volume. RT PCR was running with 3 stages: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85 °C and in the end hold temperature at 4°C then cDNA of 20ng/µl were produced.

2.4.4. Gene expression

2.4.4.1. Primer Design

The primers were designed in NCBI primer design tool by considering some important criteria like GC content, length of the primer , the size of amplicon, melting temperature, annealing temperature. Primers should be unique or very specific. To do so, sequence of each WRKY gene was aligned to find non-conserved region and allow discrimination

between different genes by using CLUSTAL W software.

Table 2. List of primers sequences used for unique amplification of WRKY genes.

Primer Name	Sequence (in 5'----> 3' order)
WRKY1_F	AGGGTAGTTCGAGTACCGGC
WRKY1_R	ACGTGCTGGACACCCTCTTA
WRKY2_F	CCGAAAACAAGTGGAGCGGA
WRKY2_R	GACGATCCGGTGGGTTTCAC
WRKY3_F1	CATGCCAAGTGCTGATGGGC
WRKY3_R1	AGGCAATGCTGCGTTTGGATT
WRKY4_F1	ACAATGAACATATTCGGGTCGGAT
WRKY4_R1	AGGCTCTCCATATCCAAGGGG
WRKY5_F	AGACCAGCAAAGAAATCTCCA
WRKY5_R	TTTCTCCAGAAACACTTATGATCG
WRKY7_F	TGCTGGTATTCCGGCAGATG
WRKY7_R	TTCCAGGATCATCGGTGGCT
WRKY8_F	TTTCCGACCACCGGAAAACG
WRKY8_R	CCCGGTATATCAGCCACCCT
WRKY9_F1	TGATGGTGGAGGAAGATGTTGTCA
WRKY9_R1	TCGTACTCGCTTTTCCTACTCTTCT

2.4.4.2. Candidate Gene

Twelve putative candidate gene involves in abiotic stress pathway, namely APX1, SOD, RBOHD, RBOHF, MCA1, NCED, ACCase, ERF1, AOS, LOXD, PAL and ICS. These genes were selected based on the information from literature and putative function of the genes. Elongation factor gene (EF1) was selected as housekeeping gene.

2.4.4.3. Quantitative/Real time PCR (qPCR)

Quantitative/Real time PCR (qPCR) was performed in a Biorad CFX thermocycler. The reaction mix contained 5 µl 2*iQ SYBR GREEN super mix, 1 µl Forward primer (3 µM), 1 µl Reverse primer (3 µM) and 3 µl cDNA (20ng cDNA) template, into a final volume of 10 µl. Elongation factor gene (EF1) was selected as housekeeping gene. Thermocycling conditions were 95^0C for 3 minutes, followed by 40 cycles of 95^0C for 10 seconds and 60^0C for 30 seconds. Relative expression was calculated using the 2-Δ ΔCt method (Livak & Schmittgen, 2001).

Table 3. List of primers sequences used for unique amplification of candidate genes for salinity tolerance in tomato

Primer Name	Sequence (in 5'----> 3' order)
SlAPX1_F	CCATTTGGAACAATCAGGCACCCG
SlAPX1_R	CGGGGCCTCCCGTAACTTCA
SlSOD_F	CCTCTCACTGGTCCACAGTCCA
SlSOD_R	AGCAGTTAACCCTGGAGGCCA
RBOHD_F1	TCAGGTCAAGCATCAAAGCCGTT
RBOHD_R1	TGGTGAAACCGCAGCACAGT
RBOHF_F1	GGAGTGGAGGGTGTGACTGGA
RBOHF_R1	GGTGCGAGTACCAGAACGCA
MCA_F1	CACTCTTTGACGTCTTTGGCG
MCA_R1	AACCATACCCATGAACCCGC
NCED1_F1	TCGAAAACCCGGATGAACAAGTGA
NCED1_R1	AACCAGAAACTTTTGGCCATGGTTC
ACCase_F2	CGCGATGAGGTTAGGTAAAAGGCA
ACCase_R2	GTCGATTCCCTTAAAAGTGGACGCA
ERF2_F1	GGAGGCGGCTAGAGCTTATG
ERF2_R1	CGGACTCGATGACTCCACAG
AOS_F1	CCGGCGGGAAGATCACGATG
AOS_R1	TCGAAAACGGCGTCGTGTGA
LOXD_F1	GCAGTACCGGACGCAACACA
LOXD_R1	CTGCAAACTTGGGCCGAGGA
ICS_F1	GGCAATAGATGCACTTCAGGCCA
ICS_R1	CGCATGGTCCCAAGACGCTTT
PAL_F1	GCTGTCAAGAACACAGTGAGCCA
PAL_R1	GGTAGGTGGAGCTGCAGGGA

2.6. Statistical Analysis

The data were analyzed by the statistical programme Genstat 15th. ANOVA was used to determine the significance difference ($P < 0.05$). And the significant results tested by Fisher's Protected Least Significant Difference test.

RESULTS

3.1. General Performance of Tomato WRKY Overexpression Lines under Salt Treatment

Many recent publications reported the involvement of WRKY transcription factors in plant defense responses. Different tomato transgenic lines overexpressing 8WRKY genes were treated with salt treatment (100 mM NaCl) and compared those responses to the control treatment (0 mM NaCl) and control genotype cv. Money Maker (MM). In presence of salt, plant height was decreased in most of genotypes (Fig.1-2) except in WRKY8-1(fig.2-l). Relative height per leaves number (internode length (cm)) also decreased under salt treatment. Most of genotypes had lower absolute fresh weight. However, dry weight and chlorophyll content were increased at salt treatment.

WRKY8-1 growth was abnormal under the control condition. WRKY8-1 had a dwarf, and it was difficult to differentiate between leaf petiole and stem (fig. 2-l). The WRKY8-1 line had a better performance under salt treatment (Fig.2-l).

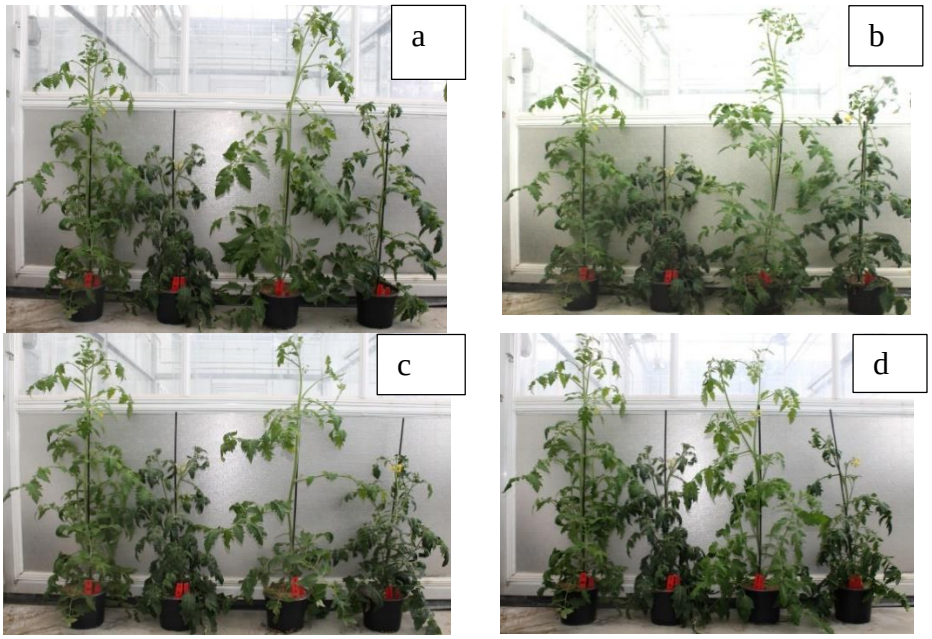

Figure 1. WRKY overexpression lines performance compared to cv. money maker (MM) under control treatment (0 mM NaCl) and salt treatment (100 mM NaCl). In each figures, from left to right: MM at control treatment, MM at salt treatment, WRKY at control treatment and WRKY at salt treatment. In each figure, WRKY overexpression line was (a) WRKY7-1; (b) WRKY 7-3; (c) WRKY9-2; and (e) WRKY9-3

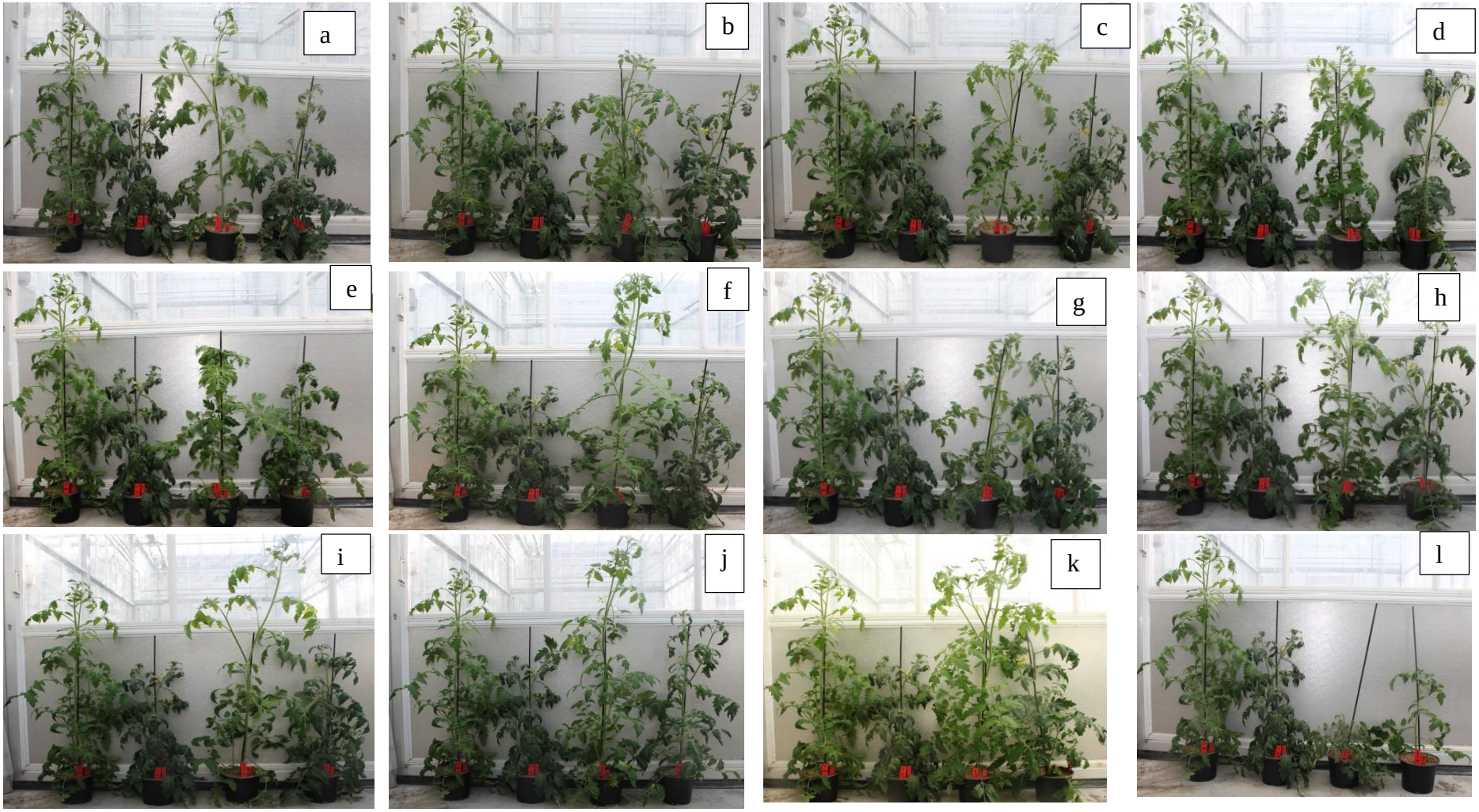

Figure 2. WRKY overexpression lines performance under control (0 mM NaCl) and salt treatment (100 mM NaCl) compared to cv. money maker (MM). In each figures, from left to right: MM at control, MM at salt, WRKY at control and WRKY at salt. In each figure, WRKY overexpression lines wes (a) WRKY1-1; (b) WRKY1-2; (c) WRKY1-3; (d) WRKY2-2; (e) WRKY3-1; (f) WRKY3-2; (g) WRKY 3-3; (h) WRKY4-1; (i) WRKY4-2; (j) WRKY4-3; (k) WRKY5-1; and (l) WRKY8-1

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3.5. *Genotype Variation of Plant Growth under Salt Treatment*

3.5.1. Internode Length

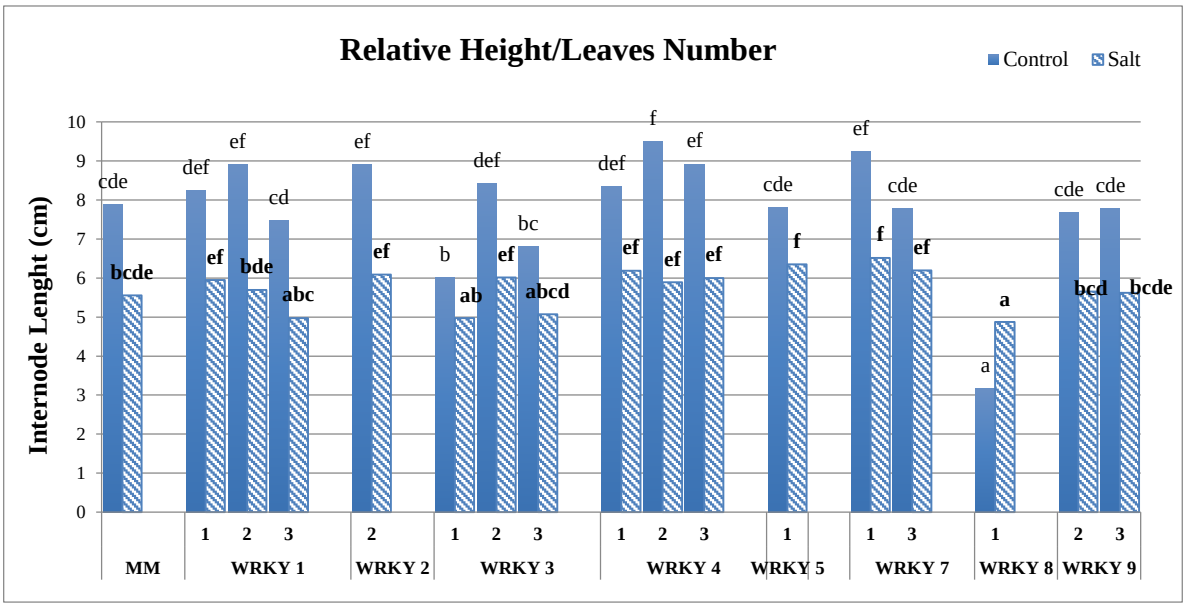

Figure 3. Effect of salt treatment (100 mM NaCl) to relative plant height/number of leaves (internode length (cm) of MM and WRKY overexpression lines. Different letters indicates significant difference at $P < 0.05$.

Internode length was measured to examine if the differences were due to internode elongation or higher leaf emergence. The internode length (cm) was calculated by dividing plant height per number of leaves. The result showed significant differences between the genotypes at both conditions ($P < 0.05$). At the control treatment, most of the overexpression lines did not show a significant difference from MM except for WRKY4-2 which had greatest internode length (9.5 cm) and WRKY8-1with the lowest internode length (3.16 cm). At salt treatment most genotypes did not show a significant different in length of internodes. Only WRKY5-1 (6.35 cm) and WRKY7-1 (6.51 cm) showed higher internode difference compared to other genotypes while WRKY8-1 had a lowest internodes (4.87 cm) although the internodes was higher compared to at control condition.(Fig. 3).

3.2.2. Plant weight

There was significant difference observed on fresh weight (FW) and plant dry weight

(DW) under control and salt treatment. The highest FW and DW under control treatment was in WRKY5-1 (FW:315.1 g; DW: 28.07 g). Meanwhile, at salt condition, the highest FW and DW was in WRKY7-1 (FW: 237.6 g; DW: 26.95 g). While WRKY8-1 had the lowest FW and DW at both treatments. (Fig.4-5).

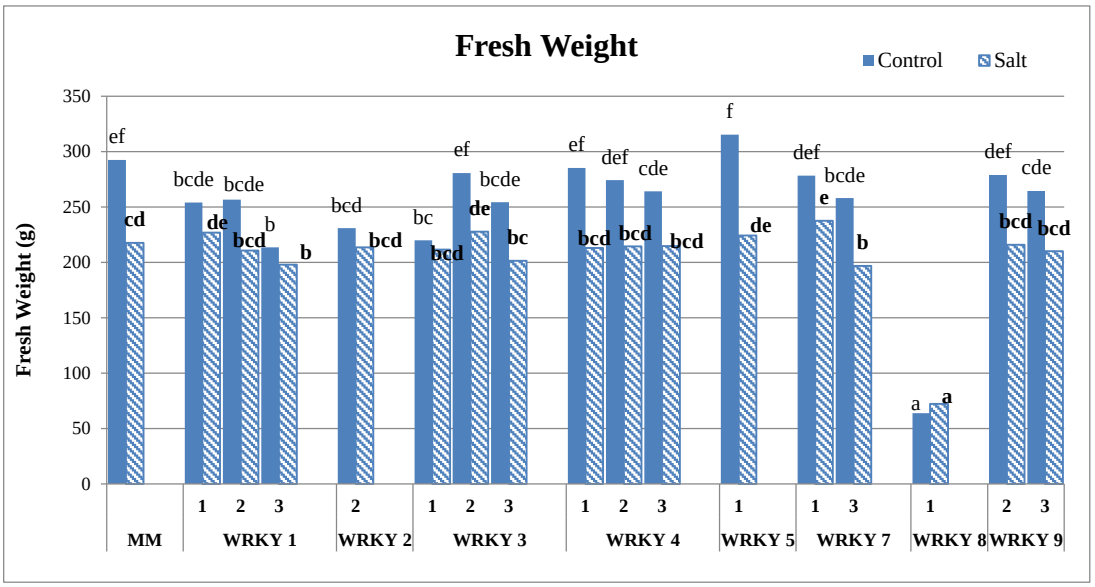

Figure 4. Effect of salt treatment (100mM NaCl) to fresh weight of MM and WRKY overexpression lines. Different letters indicates significant difference at $P < 0.05$.

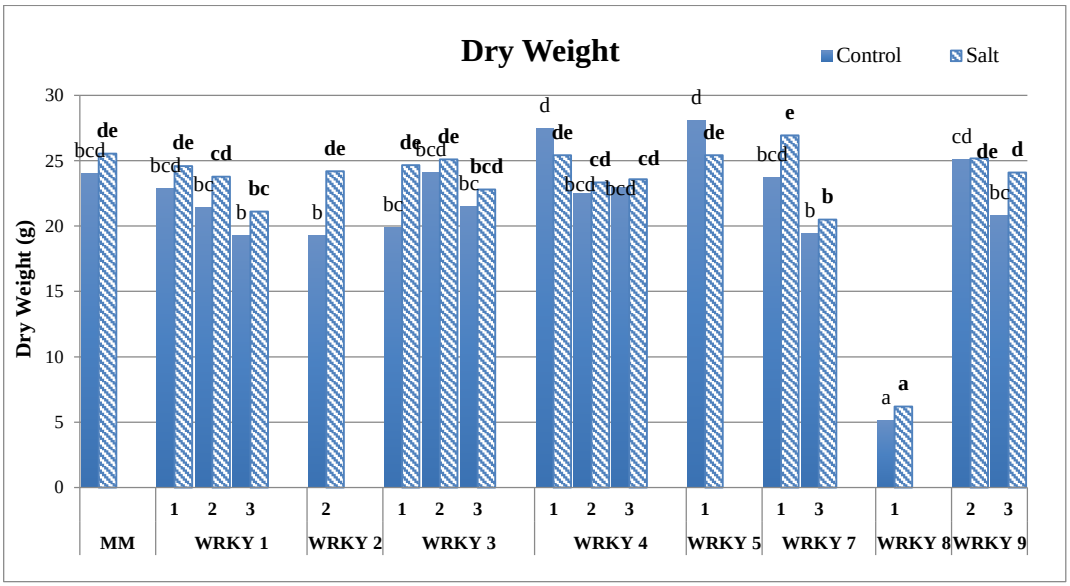

Figure 5. Effect of salt treatment (100 NaCl) to dry weight of MM and WRKY overexpression lines. Different letters indicates significant difference at $P < 0.05$.

3.2.3. Relative weight

Percentage relative fresh weight (RFW) and relative dry weight (RDW) was calculated by

normalizing the fresh weight and dry weight of each genotype at the salt treatment to the the fresh weight and dry weight at control treatment. The result presented that most of genotypes had the RFW less than 100% with the exception of WRKY8-1which had the RFW 112.99%. Conversely, WRKY5-1 had the lowest RFW with (17.17%).

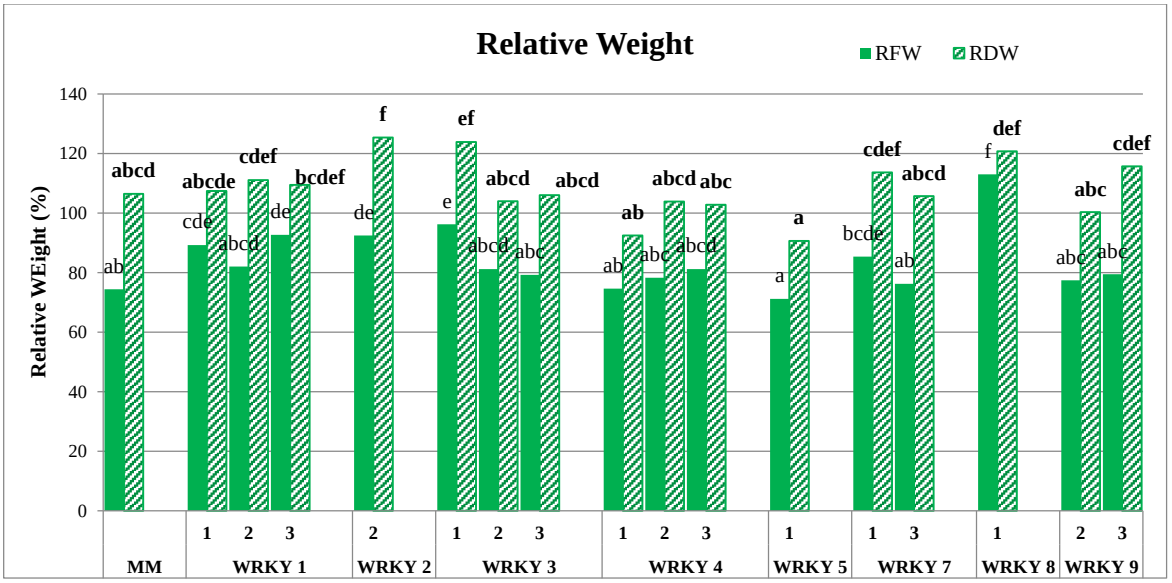

Figure 6. Effect of salt treatment (100mM NaCl) to percentage of RFW and RDW of MM and WRKY overexpression lines. Different letters indicates significant difference at $P < 0.05$.

In contrary with RFW, most of overexpression lines RDW was higher than 1. The RDW of WRKY2-2 and WRKY3-1 were slightly higher compared to other genotypes (125.4% and 123.9%, respectively). WRKY5-1 had the lowest RDW (90.6%).(Fig.6.).

3.2.4. Chlorophyll content

In general, chlorophyll content at the salt treatment was higher than chlorophyll content at the control treatment. The result of chlorophyll content of genotypes at both treatments showed a significant different ($P < 0.05$). Most of overexpression lines at the control treatment have a similar or slightly higher chlorophyll content compared to MM. Chlorophyll content in control conditions varied from 40.3 to 45.32. WRKY8-1 had significant lower chlorophyll content (40.3) than MM. The chlorophyll content was increased at salt treatment. Most of overexpression lines had less chlorophyll content compared to MM. WRKY1-2 had slightly higher chlorophyll content (57.84).

WRKY3-1 and WRKY8-1 had significant lower chlorophyll content (47.45 and 46.91, respectively) compared to MM. (Fig.7.)

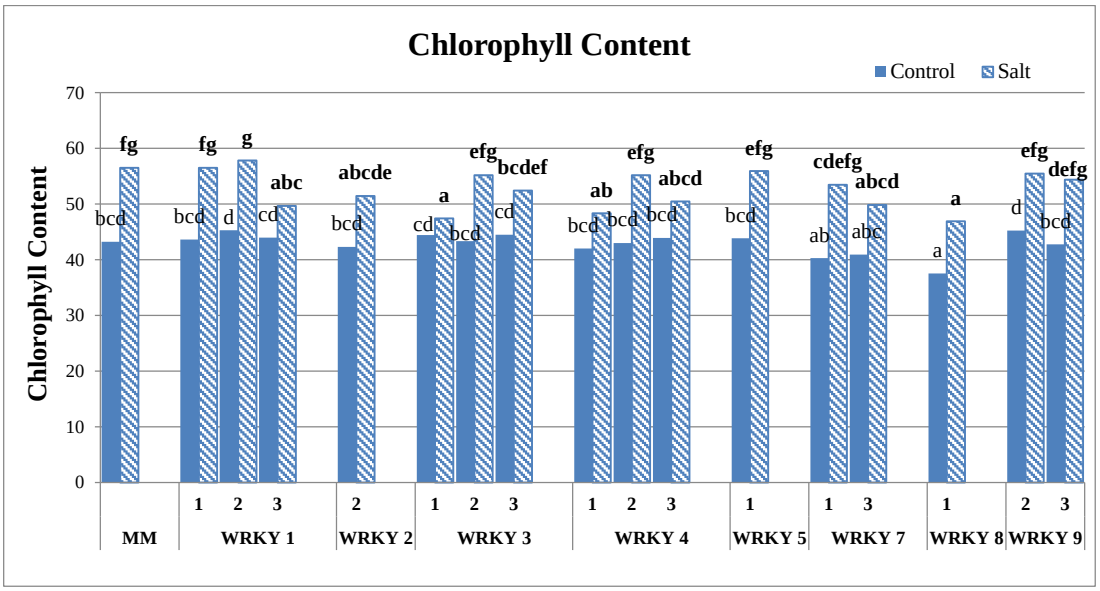

Figure 7. Effect of salt treatment (100mM NaCl) to relative chlorophyll content of MM and WRKY overexpression lines. Different letters indicates significant difference at $P < 0.05$.

3.3. Genotypes Variation of Electrolyte Leakage (EL)

Paraquat (*N*,*N*′-dimethyl-4,4′-bipyridinium dichloride**)** is a one of the most widely used herbicides in the world. This chemical is widely used to induce oxidative stress for testing the sensitivity of plant genotype to oxidative stress. Paraquat can induce plant to produce ROS like superoxide radical, singlet oxygen hydrogen peroxide and hydroxyl radical. Those reactive compounds cause the degradation of proteins, pigments, lipid peroxidation, and affect the plant cell metabolism which leads to cell death (H. R. Lascano, Gomez, Casano, & Trippi, 1998; H. Ramiro Lascano, Gómez, Casano, & Trippi, 1999). Electrolyte leakage (EL) measurements is one of the indications of the cell destruction due to oxidative stress.

The application of two different concentrations of paraquat (1 µM and 0.5 µM) and three different time-point measurements showed there were significant differences of EL among genotypes in all concentrations and at all different time points. The application of 1 µM paraquat had a more pronounced effect on EL compared to 0.5 µM paraquat at the same

time-point measured. Comparing the time measurement, the variation of EL among genotype showed that the coefficient of variation and standard error were higher at 24 hours measurement compared to 48 hours and 72 hours measurement. (Appendix 7.2).

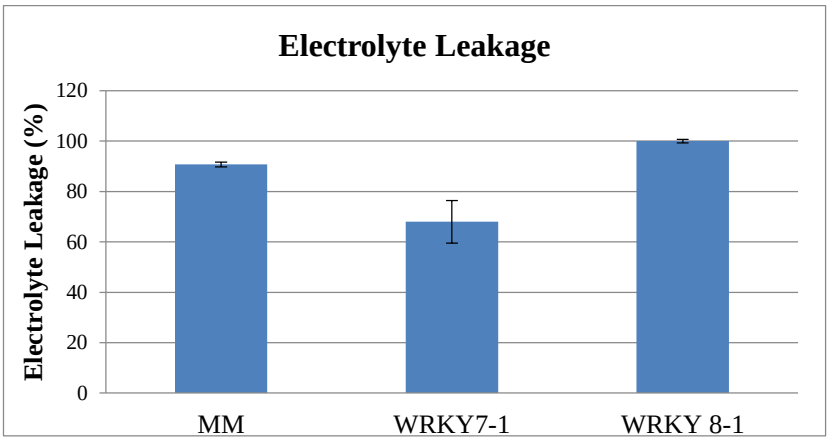

Figure 8. Percentage of EL of MM, WRKY7-1 and WRKY8-1at 72 hours after application of 1 µM paraquat. The bar represented standard error.

After 72 hours of 1 µM paraquat application, the highest percentage of EL was observed in WRKY8-1(100% EL) which indicated that WRKY8-1 had the highest stress damage caused by ROS production. It indicated that WRKY8-1 had Meanwhile, WRKY7-1 had the lowest EL (67.98%). Likewise, it also produced lower EL at 0.5 µM paraquat concentration (Appendix 7.2) which indicated that WRKY7-1 had a lower stress damage compared to other genotypes after paraquat applications. (Fig.8).

3.3. Genotypes Variation of Ion Content Analysis

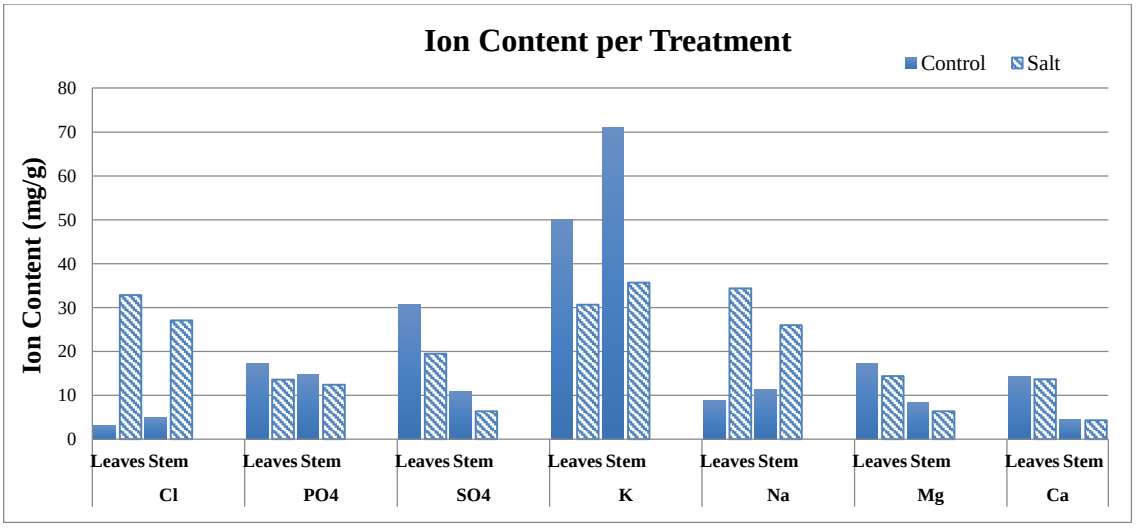

Figure 9. Ion content (mg/g) in leaf and stem of all genotypes under control and salt (100 mM NaCl).

Ion chromatography analysis was applied to analyze the concentration of Chloride (Cl^-), Sulfate (SO_2^{4-}), PO_4^{3-} (Phosphate), Na^+ (Sodium), K^+ (Potassium), Mg^{2+} (Magnesium) and Ca^{2+} (Calcium) in plant leaves and stem as an indicator of sensitivity to salt application. The total ion content of each treatment revealed that ion content in leaves and stem had the same trend. The application of salt greatly increased the accumulation of Cl^- and Na^+. The accumulation of PO_4^{3-} and Mg^{2+} in leaves and stem at the salt treatment were slightly less than the control treatment while Ca^+ was similar in control and salt treatments. SO_2^{4-} and K^+ in leaves and stem were greatly decreased under the salt treatment. However, compared to previous study (Sunarti, 2012) the content of SO_2^{4-} and K^+ in leaves and stem at control condition was much higher. (Fig.9).

3.4.1. Genotype variation of ion accumulation on plant leaves and stem under salt treatment

3.4.1.1. Chloride content

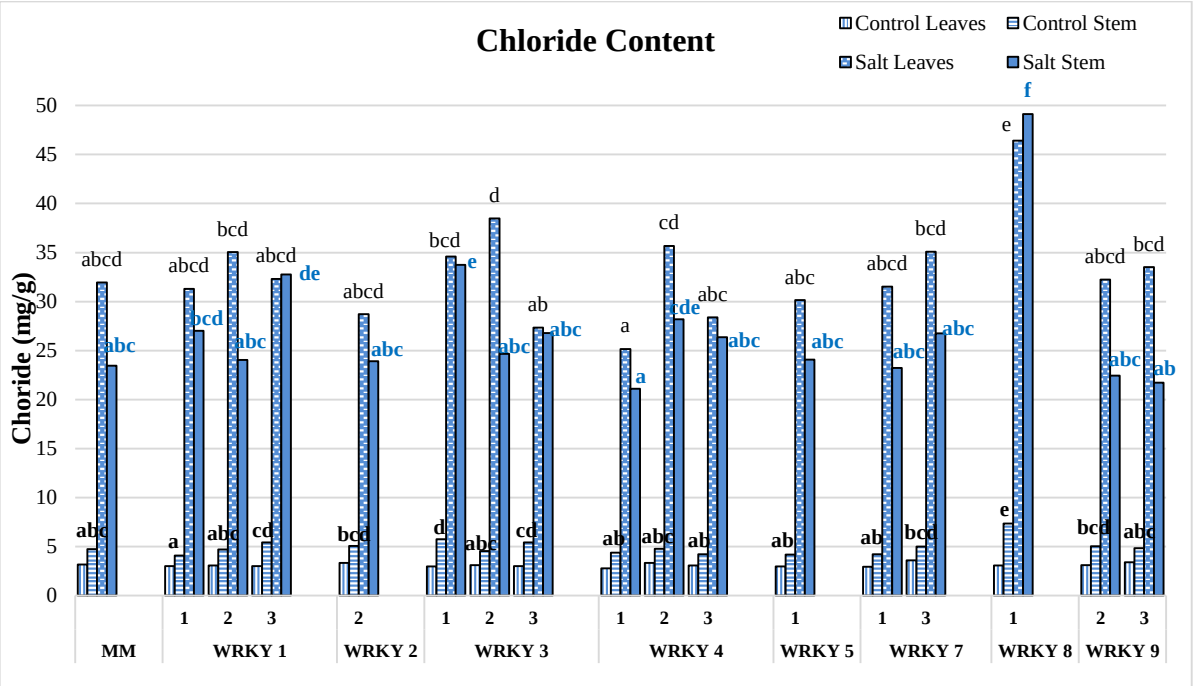

Figure 10. Chloride content (mg/g) in leaves and stem of MM and WRKY overexpression lines under control and salt treatment. Different letters indicates significant differences ($P < 0.05$).

Based on genotypic differences, the accumulation of Cl^- gave significant differences in leaves at the salt treatment and in stem at control and salt treatment. At the salt treatment WRKY8-1 had the highest Cl^- accumulation (leaves: 46.41 mg/g; stem: 49.14 mg/g compared to MM (leaves: 31.96 mg/g; stem: 13.44 mg/g) and other overexpression lines. While, WRKY4-1 had the lowest Cl^- content in its leaves and stem (25.16 mg/g and 21.10 mg/g, respectively). However, the accumulation of Cl^- in other overexpression lines did not show significant differences compared to MM (Fig.9).

3.4.1.2. Potassium Content

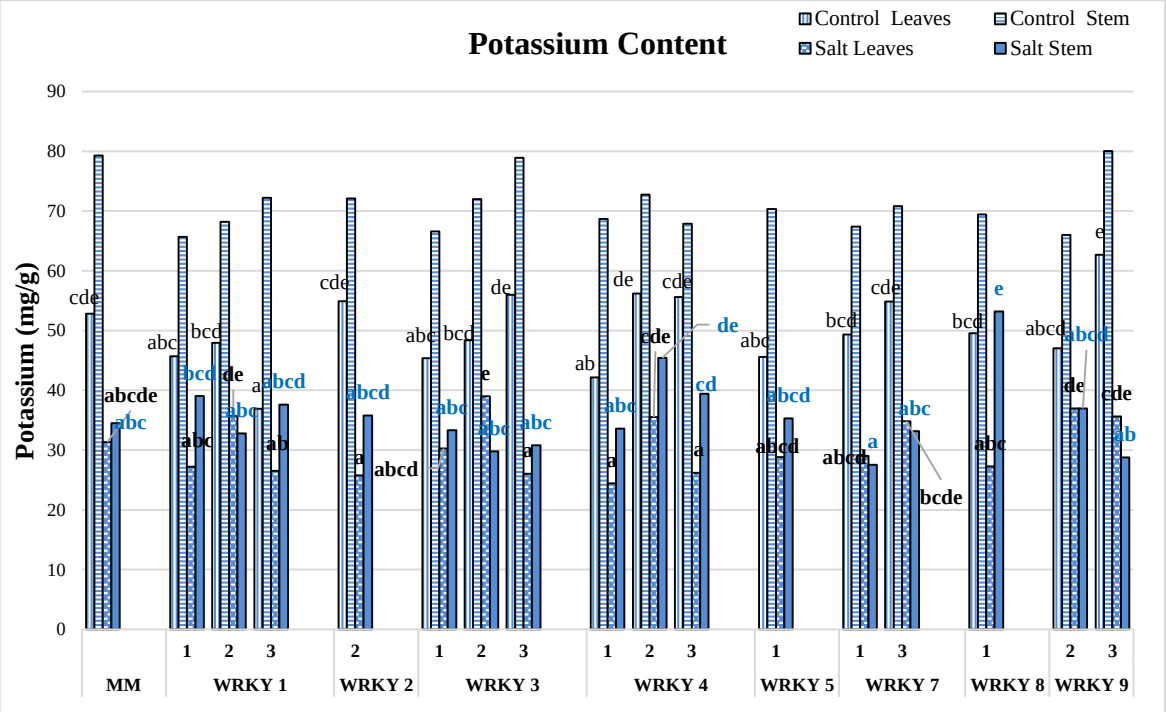

Figure 11. Potassium content (mg/g) in leaves and stem of MM and WRKY overexpression lines under control and salt treatment. Different letters indicates significant differences ($P < 0.05$).

The accumulation of K^+ in plant decreased with salt treatment. The different genotypes gave a significant difference in K^+ content in leaves in control treatment and at the stem in salt treatment. At the control and salt treatments, leaves accumulated higher of K^+ compared to K^+ in stem. Yet some genotypes had higher K^+ in the stem compared to the leaves. In the leaves at the salt treatment, leaves of WRKY3-2 can keep K^+ content higher (36.97 mg/g) compared to MM (31.35 mg/g). K^+ accumulation in stem was highest

in WRKY8-1 (53.20). Thus, MM was accumulated 34.52 mg/g in the stem. This content was not significant difference with overexpression lines. (Fig.11).

3.4.1.3. Sodium Content

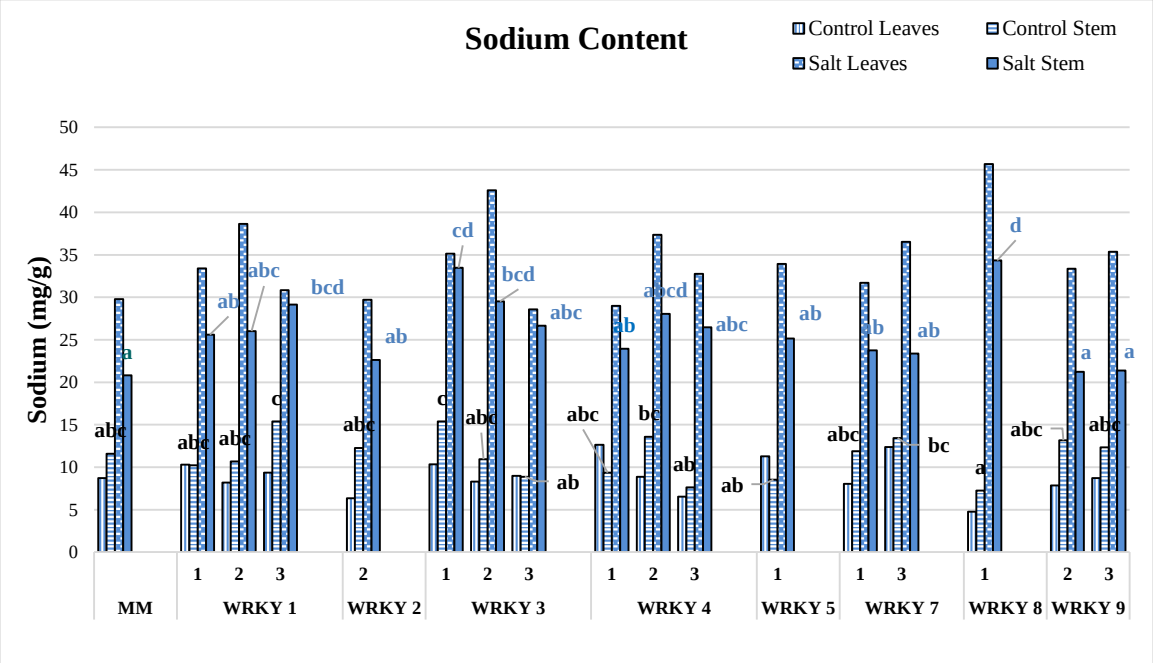

Figure 12. Sodium content (mg/g) in leaves and stem of MM and WRKY overexpression lines under control and salt treatment. Different letters indicates significant differences ($P < 0.05$).

The accumulation of Na^+ in leaves did not show any significant differences between genotypes at both control and salt treatment. On the other hand, there were significant differences among genotypes in stem at both treatments. In general, Na^+ content in leaves was higher compared to Na^+ content in stem. In stem at salt treatment, the highest accumulation of Na^+ content was found in WRKY8-1(23.39 mg/g) and the lowest content of Na^+ was in MM, WRKY9-2, and WRKY9-3 (20.81 mg/g, 21.24 mg/g, and 21.38 mg/g, respectively). (Fig.12).

3.5. Gene Expression

The transgene expression of each of the genotypes under control condition was calculated relative to gene expression of the respective endogenous genes MM. Expression data were normalized to the the values of the endogenous genes observed in

MM. The WRKY transgenes were highly expressed in the majority of lines. Line WRKY1-3 was highly expressed while WRKY1-1 and WRKY1-2 had lower expression. However, the level of WRKY3-2 expression was lower compared to WRKY3-1 and WRKY 3-3. Similarity, WRKY4-1 and WRKY4-2 were higly expressed while WRKY4-3 was lower expressed. Moreover, others WRKY reminded highly expressed. The highest expression among genotypes was observed in WRKY9-2 and WRKY9-3 with the value of 314.10 and 295.49 respectively (Table 4.).

Table 4. Gene expression of WRKY overexpression lines realative to MM

Genotype	Gene Expression Relative to MM
WRKY1-1	0.34 ±0.03
WRKY1-2	0.57±0.12
WRKY1-3	6.42 ±0.03
WRKY2-2	2.0 ±0.48
WRKY3-1	38.35 ±2.72
WRKY3-2	0.78 ±0.00
WRKY 3.3	29.07 ±4.95
WRKY4-1	1.48 ±0.35
WRKY4-2	1.42 ±0.45
WRKY4-3	0.64 ±0.04
WRKY5-1	8.18 ±4.06
WRKY7-1	3.39 ±0.03
WRKY 7.3	3.87 ±0.04
WRKY8-1	118.10 ±21.06
WRKY 9.1	314.10 ±18.55
WRKY9-2	295.49 ±44.59

3.5.1. Gene expression under salt treatment

The gene expressions of 12 genes corresponding to pathways related to salt stress adaptation were observed. Because of time limitation, only three WRKY overexpression lines were included in this experiment (WRKY3-3, WRKY7-1 and WRKY8-1). The selection was based on the result of growth parameter, EL and ion content; and information of homologous WRKY function (mostly form Arabidopsis). The gene expression was a relative expression of each genotype under salt treatment relative to gene expression of MM under the control treatment. Therefore the value of MM (at control treatment) was 1.

3.5.2. Genes relate to ROS scavenging pathway

APX and SOD are enzymes that are involved in ROS scavenging mechanism. At the salt treatment, APX1 gene in all genotypes were highly expressed compared to control treatment. APX1 in WRKY3-3 had slightly lower expressed compared to MM. Meanwhile the lowest APX1 expression was found in WRKY8-1. (Fig.13).

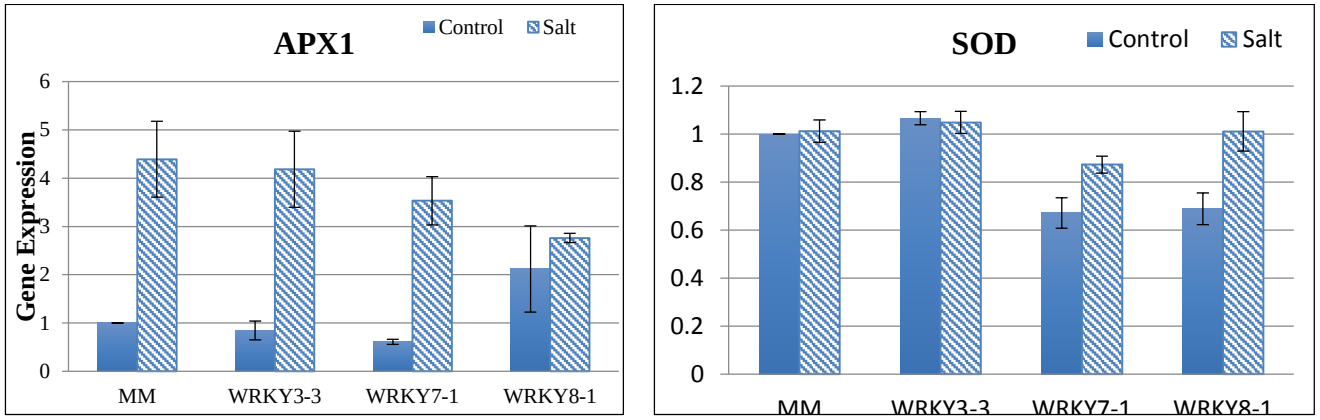

Figure 13. Gene expression level of APX1 and SOD gene in MM, WRKY 3.3, WRKY7-1 and WRKY8-1at control (0 mM NaCl) and Salt (100 mM NaCl) treatments. The bar represented standard error.

Furthermore, at control and salt treatment, gene expression of SOD in MM and WRKY3-3 were similar. In WRKY7-1, expression of SOD was slightly higher at the salt treatmentWRKY7-1). Moreover, in WRKY8-1 the level of SOD was increased at salt treatment (Fig. 13).

3.5.3. Genes relate to the NADPH pathway

NADPH oxidase is a major source of ROS. It converts the superoxide anion ($O_2^{\cdot -}$) to other ROS, such as per hydroxyl radicals, hydroxyl radicals and hydrogen peroxide (Foreman et al., 2003). Plant Respiratory Burst Oxidase Homologues (RBOHs) gene family is an enzymatic subunit of the plant NADPH oxidase which responsible to encode plasma membrane-associated NADPH oxidase to produce a signal transduction-associated ROS in the cell during environmental stress. (Fig.14).

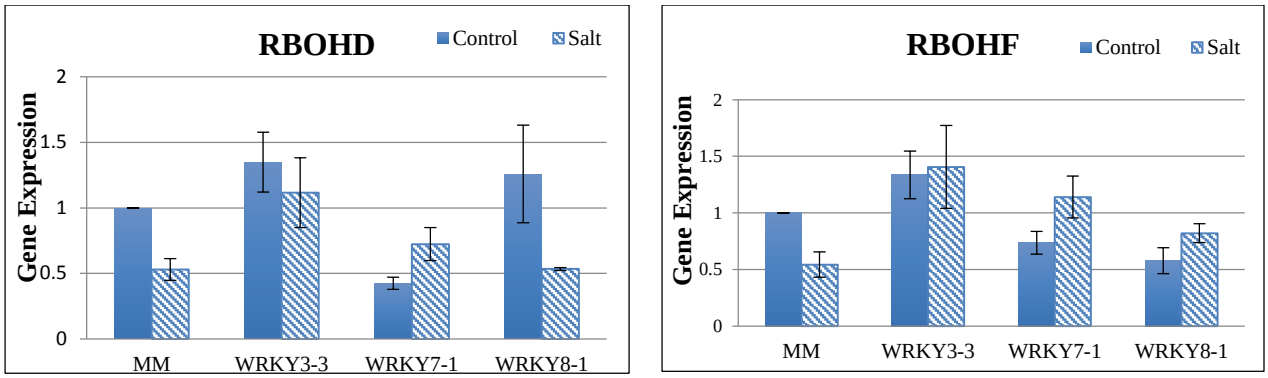

Figure 14. Gene expression level of RBOHD and RBOHF in MM, WRKY 3.3, WRKY7-1 and WRKY8-1at control (0 mM NaCl) and Salt (100 mM) treatments. The bar represented standard error.

The level of RBOHD expression in MM and WRKY8-1was lower at salt treatment compared to control condition. In WRKY 3.3, different treatment did not give a high difference in the level of RBOHD expression though the expression level was slightly higher at the control treatment (salt: 1.12; control: 1.35). Moreover, in WRKY7-1 RBOHD was higher expressed under salt treatment even the value still less than MM in control. Furthermore, RBOHF expression were lower in MM under the salt treatment. While, the expression level of RBOHF in overexpression lines were increased at salt condition. Similarly, the level of RBOHF gene expression in WRKY3-3 was slightly higher at salt treatment (salt: 1.41 and control 1.34). Whereas, in WRKY7-1 under the salt treatment, RBOHF was more expressed compared to control condition (1.14 times of MM control). Meanwhile, WRKY8-1 expressed slightly higher RBOHF under salt condition thought the value still less than MM control (0.82). (Fig.14).

3.5.4. Gene mediates cell death regulation

MCA1 gene has been identified for coding for a metacaspase involved in the induction of cell death (Mazzoni & Falcone, 2008). MCA1 in WRKY3-3, WRKY7-1 were highly expressed at both treatments with almost similar value between the expression at salt and control. MM at salt treatment had slightly higher MCA1 expression compared to the control. However, in WRKY8-1 the level of gene expression at salt condition lower compared to at control condition. (Fig.15).

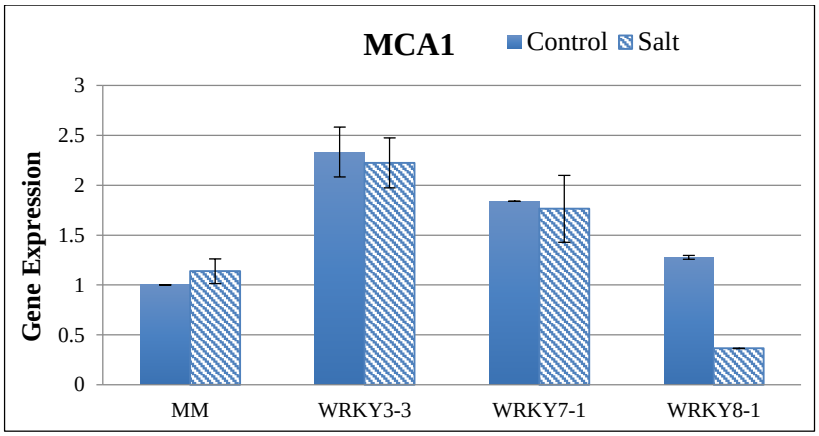

Figure 15. Gene expression level of MCA1 in MM, WRKY 3.3, WRKY7-1 and WRKY8-1at control (0 mM NaCl) and Salt (100 mM) treatments. The bar represented standard error.1

3.5.5. Genes relate to hormonal pathways

3.5.5.1. Abscisic Acid Pathway

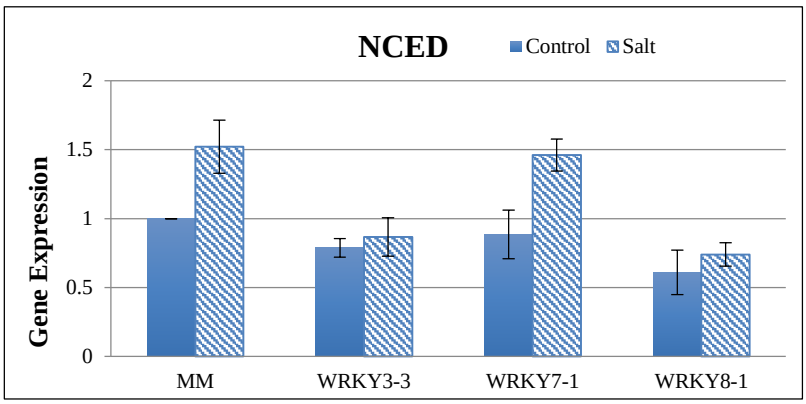

Figure 16. Gene expression level of NCED in MM, WRKY 3.3, WRKY7-1 and WRKY8-1at control (0 mM NaCl) and Salt (100 mM) treatments. The bar represented standard error.

Abscisic acid is a plant hormone that is involved in plant stress response. NCED (*9-cis-epoxycarotenoid dioxygenase*) is one of the genes that contributes in ABA biosynthesis. NCED gene in WRKY3-3 and WRKY8-1 had a low expression at control and salt treatment. Whereas, in MM (1.52) and WRKY7-1 (1.46), NCED had higher expression under the salt treatment. (Fig.16).

3.5.5.2. Ethylene Pathway

Ethylene is a plant hormone that is involved in the stress response mechanism. It protects plants against biotic and abiotic stress via cross talk with other hormones such as

jasmonic acid, salisylic acid or abscisic acid by synergetic or antagonistic pathway. ACC synthase (ACCase) is the gene that catalyzes the synthesis of ACC precursor of ethylene. Observed result presented ACCase expression under salt treatment markedly increased in WRKY3-3 (41.1) and WRKY7-1 (4.30). Likewise, the level of ACCase in WRKY8-1 was higher at control compared to salt treatment. Nevertheless, the level of ACCase expression in WRKY8-1under salt condition was higher than MM (6.63). (Fig.17)

Another gene that involves in ethylene signaling s is ERF1 (ethylene-responsive element binding factor 1). ERF1 gene is a member of a novel family of transcription factors (ERFs) that has a function in regulation of extracellular signals and it regulates a subset of GCC box–containing stress response genes (Fujimoto et al, 2000). The result of the experiment showed under salt condition, ERF1 was highly expressed in all genotypes. The highest expression level was found in WRKY3-3 (9.85). Although, in WRKY8-1the ERF1 expression level under salt treatment was slightly decreased compared to control, but the level of expression still higher than 1 (5.26). (Fig.17)

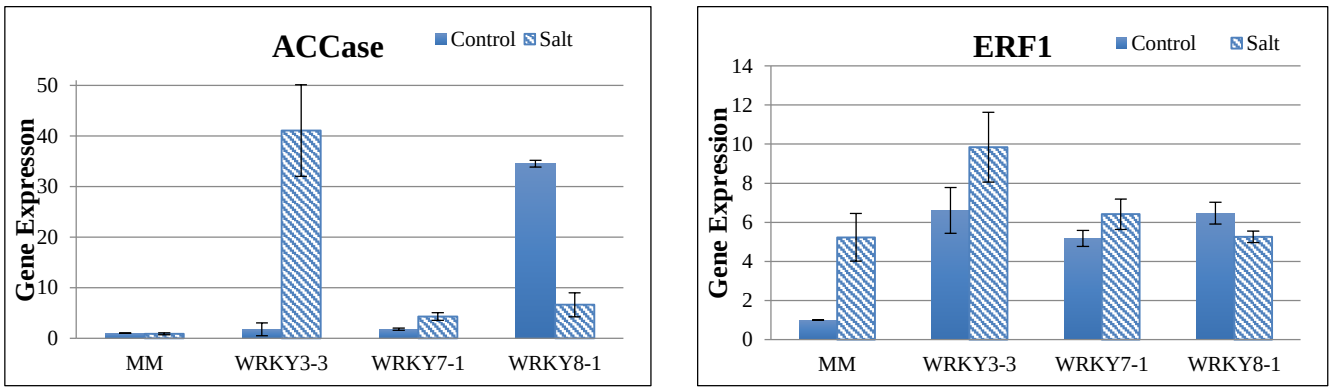

Figure 17. Gene expression level of ACCase and EFR1 in MM, WRKY 3.3, WRKY7-1 and WRKY8-1at control (0 mM NaCl) and Salt (100 mM) treatments. The bar represented standard error.

3.5.5.3. Jasmonic Acid Pathway

Jasmonic acid (regulates a pivotal role in some physiological processes, flower development, and defense mechanism against biotic and abiotic stress (Farmer & Ryan, 1990). *Allene oxide synthase* (AOS; hydroperoxide dehydratase) is the gene that catalyzes the production of unstable allene epoxides that cyclize to form cyclopentenone

acids, the precursors for JA (Mueller, 1997). AOS gene was slightly expressed in WRKY3-3 at control condition (1.26). In all genotypes, AOS gene expression were lower at salt treatment. However, in general this gene was poorly induced in all genotypes under control and salt treatments.

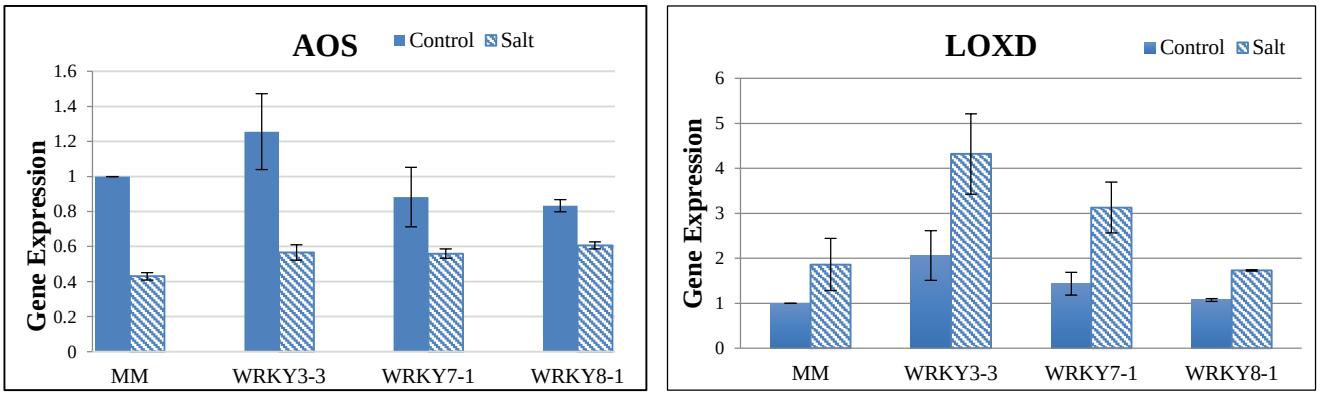

Figure 18. Gene expression level of AOS and LOXD in MM, WRKY 3.3, WRKY7-1 and WRKY8-1at control (0 mM NaCl) and Salt (100 mM) treatments. The bar represented standard error.

The other gene that involves in JA biosynthesis and signalling is LOXD (Lypoxygenase). This gene is well known as a defense-related gene because it is up-regulated in leaves in response to wounding that activates jasmonate mediated defense mechanism. The salt stress induced higher expression of LOXD gene in all genotypes. Under salt treatment the highest expression level of LOXD was found in WRKY3-3 (4.31). Whereas, LOXD in WRKY7-1 (3.13) was lower expressed compared to this expression in WRKY3-3. (Fig.18).

3.5.5.4. Salicylic acid pathway

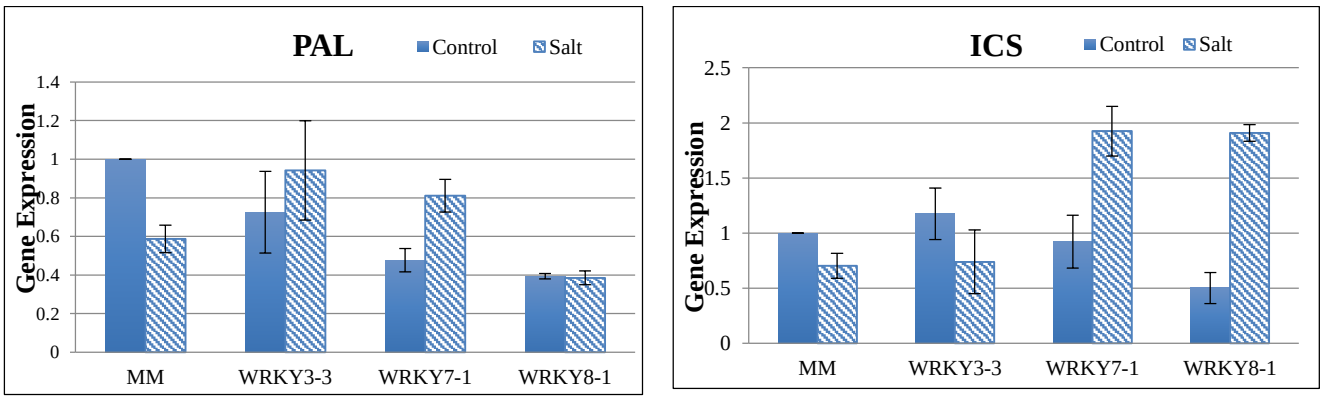

Figure 19. Gene expression level of PAL and ICS in MM, WRKY 3.3, WRKY7-1 and WRKY8-1at control (0 mM NaCl) and Salt (100 mM) treatments. The bar represented standard error.

Salicylic acid (SA) is an important hormone in regulating plant defense mechanism. Plants synthesize SA from two pathways, firstly, SA is synthesis from cinnamate produced by the activity of phenylalanine ammonia lyase (PAL) enzyme. PAL gene encrypts the synthesis of PAL enzyme to the upstream component of SA biosynthesis and is induced under a variety of biotic and abiotic stress treatments. SA is formed from cinnamate via benzoate or *o*-coumarate depending on the position of the hydroxylation of the aromatic ring takes place before or after the chain-shortening reactions (Klambt, 1962). The result showed that PAL gene in all genotypes induced under salt treatment except WRKY8-1, in contrast to MM where it was repressed.

In the second pathway SA is synthesized from chorismate through two reactions catalyzed by isochorismate. This pathway involves isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) to produce a bulk SA. ICS catalyzes the synthesis of isochorismate from chorismate and IPL catalyzes the conversion of SA from isochorismate (Serino et al., 1995). Under salt condition ICS was up regulated in WRKY7-1 (1.93) and WRKY8-1(1.90). (Fig.19.)

DISCUSSION

4.1. Growth Parameters of Tomato WRKY Overexpression Lines under Control and Salt Treatment

Salinity is one of the most severe abiotic stresses limiting agricultural production. Application of 100 mM NaCl over several weeks to tomato WRKY overexpression lines affected plant growth parameters. Most genotypes showed reductions in plant height and relative fresh weigh in response to salt treatment. However WRKY8-1 had better performance under salt treatment, plants were taller and no leaves overgrown. This might due to a suppressing effect of salt on the pleiotropic effect of WRKY 8.

The taller and longer internodes for some genotypes like WRKY4-2, WRKY5-1and WRKY7-1 might indicate altered GA signaling which involved in stem elongation. There no other pleiotropic effects for these lines since we have the similar phenotypic performance. WRKY3-1 and WRKY3-3 were smaller than WRKY3-2 and MM plants. WRKY3-1 and WRKY3-3 plants exhibited small plant and extensive branching. The WRKY3 lines overexpress (SlWRKY6) is homologous with *AtWRKY6.* Overexpression of *AtWRKY6* plant resulted in small and stunted plants, altered leaf morphogenesis and change in flowering time (Robatzek & Somssich, 2002).

The higher chlorophyll content in leaves under salt treatment might also be related to the accumulation of carbohydrate and sugars in leaf (Saab, 1990) this probably also related to the decreased of cell expansion of the leaves at salt treatment. Higher carbohydrate and sugar in leaf might be also related to DW at salt treatment. Besides, higher DW under salt stress connected to the stem characteristics. Stem was thicker and more rigid in compared to control plants, which may reflect a greater deposition of lignin in the cell walls (Christensen, Bauw, Gjesing Welinder, Van Montagu, & Boerjan, 1998), vascular tissues and/or more extensive xylem development. Under salt stress, the higher lignified in tracheary element may compensate for the reduction in water permeability that is synchronize with greater solute selectivity during xylem sap loading (Sánchez-Aguayo, Rodríguez-Galán, García, Torreblanca, & Pardo, 2004).

4.1. Performance of Tomato WRKY Overexpression Lines under Salt Treatment

Interesting phenotypes was found in WRKY5, WRKY7 and WRKY8. WRKY5-1 had higher absolute plant height but not in relative growth. Meanwhile, WRKY7 (SGN WRKY11) showed taller, higher FW and DW compared to MM both in control and salt conditions. This genotypes showed indications of salt tolerance. WRKY7 (*SlWRKY11*) has high sequence similarity with WRKY8, but the lines WRKY 7-1 and 7-3 showed extremely different performance in morphological aspects compared to WRKY 8-1. These differences in phenotype indicate that even slight changes in protein sequence of promoter may change in structure of chromatin and have a significant effect on TFs binding to downstream promoter sequences and have to be further explored.

The line WRKY 8-1(SGN WRKY10) showed more severe dwarfing/stunting in plants growing under control conditions. A homologue of this gene is *AtWRKY22;* overexpression of *AtWRKY22* results in defective morphology. Mutant Arabidopsis plants were stunted, and showed compact growth, narrow leaves, and partial sterility. The young siliques were undeveloped and empty and the final seed content was reduced compared to the control plants (X. Zhou, Jiang, & Yu, 2011). WRKY 8-1 also produced a very low number of seeds, and that was the reason that no more independent lines were available for comparative analysis. While in salt treatment, plants had better growth with taller, longer internode, thicker and more rigid stems. This maybe a result of suppressive effect of salt stress on the growth defects caused by WRKY8 overexpression and need to be further examined.

4.2. Genotype Differences in Electrolyte Leakage and Ion Content

Oxidative stress can be triggered by a wide range of environment conditions such as UV-light, salinity, drought, heavy metals, chilling, oxygen shortage, and nutritional deprivation (Rizhsky, Liang, & Mittler, 2002). Oxidative stress tolerance is a desired trait as is a components of tolerance to different abiotic stresses (Munns & Tester, 2008).

Paraquat is an effective chemical for inducing oxidative stress and causing damage to plant cell membranes. The result of membrane damage can be monitored by measurement of electrolyte leakage (EL). Paraquat application had a significant effect on leaf EL percentage, which indicated that the leaf was under oxidative stress. The highest EL was noted in WRKY8-1, indicating that this line was sensitive to oxidative stress that caused by paraquat application. This coincides with experiments with an Arabidopsis WRKY gene with high sequence similarity, *AtWRKY15*, as its overexpression in Arabidopsis resulted in a higher susceptibility to oxidative stress compared with wild type (WT) plants. This stress response was linked to a stimulation of endoplasmic reticulum-to-nucleus communication and a disruption of mitochondrial stress responses under salt-stress conditions (Vanderauwera et al., 2012). However, compared to WT plants the *AtWRKY15* overexpression showed increased biomass and salt stress sensitivity, in contrast to our results.

The WRKY 7 lines (WRKY 7-1 and WRKY 7-3) had the lowest EL during the paraquat experiment. Its homologous gene in Arabidopsis is co-expressed with various oxidative stress tolerance genes. Among them *AtWRKY22* was highly expressed under H_2O_2 stress and served as a tolerance mechanism (Zhou et al., 2011)). The *AtMPK3* (ARABIDOPSIS THALIANA MITOGEN-ACTIVATED PROTEIN KINASE 3)/MAP3 kinase appears to be co-expressed with *AtWRKY22* (http://string-db.org/newstring_cgi/show_network_section.pl). MPK3 signaling through the MAP kinase cascade can lead to cellular responses including cell division and differentiation as well as responses to various stresses (osmotic shock, oxidative stress, response to cold, and anti-pathogen responses (Sinha, Jaggi, Raghuram, & Tuteja, 2011).

Ion content analysis is an effective selection method for genotype tolerance to ionic stress. However, none of the WRKY lines significant differences except WRKY8-1, which had both higher Na^+ and Cl^- contents compared to MM which may be related to the ion compartmentalization of Na^+ and Cl^- in its vacuole. Taking into account that the production ROS may affect ion content. In Arabidopsis under salt stress, ROS was produced by both AtrbohD and AtrbohF which has function as signal molecules to

regulate Na^+/K^+ homeostasis, thus improving the salt tolerance of Arabidopsis (Ma et al., 2011).

4.2. Gene Expression of WRKY Overexpression Lines

Gene expression analysis was done to see the expression of transgene expression of each of the genotypes under control condition. The expression was assesed relative to gene expression of the respective endogenous genes of MM. Most of the transgenes were overexpressed compared to the WT (MM). The 35S Promoter is a very strong constitutive promoter. In dicot plant like tomato, the use of its promoter in plant transformation induces high levels of gene expression. Most of WRKY lines exhibited phenotype performance that correlated with the relative level of expression of the transgene. For example endogenous gene expression of WRKY3-1 and WRKY3-3 were higher compared to the expression level of WRKY3-2 and the expression level of the native gene (MM). Besides. WRKY3-2 expression level also lower than native gene expression. Expression variation between the different independent lines might be due to position effects, DNA methylation and post transcriptional silencing (Kooter, Matzke, & Meyer, 1999).

4.2. Gene Expression of WRKY Overexpression Lines under Salt Treatment

The gene expression of several genes involved in salt stress acclimation and tolerance were studied in MM, WRKY3-3, WRKY7-1, and WRKY8-1 under control and salt treatments. This study demonstrated that salt stress caused variations in the expression levels of genes encoding ROS scavenging, NADPH pathway, plasma membrane cell death, and hormonal pathways.

Salt stress induced APX (Shalata & Tal, 1998), but no significant differences were observed between the genotypes. Not significant changes in SOD expression were observed under stress, with WRKY7-1 and WRKY8-1 exhibiting lower expression. Despite WRKY7-1 had high oxidative stress tolerance, these results indicate that the

difference might be because of other genes or TFs that activated different pathways involved in the stress response mechanism, such as chaperones function, osmoprotectants, and factors controlling water and ion movement (Vinocur and Altman, 2005). The overexpression of *DgWRKY1* in tobacco resulted in enhanced tolerance to salt stress but no significant difference in the stress-related gene expression was found between overexpression line and WT. WRKY may work with other regulators to promote the expression of stress-responsive genes especially under stress condition (Q. L. Liu et al., 2013).

The expression of several pathway genes that regulate the salinity stress-tolerance mechanism in WRKY7-1 was quite similar to expression in the MM control. Higher levels of ABA and JA (LOXD) were induced under salt treatment in both lines. In contrast to the MM plants, genes related to ICS (SA) and ethylene (ACC and ERF1) were highly expressed in WRKY7-1 In tomato cell suspension, oxidative stress induces SA-induced the cell death by the activation of MAPKs and cysteine proteases that mediates the cell death signaling and ET can accelerate the process only in cells exposed to high salinity (Poor, Kovacs, Szopko, & Tari, 2013).

In WRKY3-3, the expression differed from that seen in the other WRKY lines. Under salt conditions, WRKY3-3 showed a very high expression of ethylene and high expression of JA (LOXD), but low expression of ABA and SA. Cheng et al (2013) revealed in Arabidopsis, salt stress induction ERF1 was enhanced by ET-JA signaling and suppressed by ABA. ERF1 acts downstream of the intersection between ethylene and jasmonate pathways and suggest that this transcription factor is a key element in the integration of both signals for the regulation of defense response genes. Moreover, WRKY3-3 showed senescence phenotype and high branching. Senescence is regulated by internal ET, JA and ABA (Gan, 2003) while shoot branching involves various hormonal pathways, of which auxin is the dominant, but ethylene might also be involved as it interacts with auxin (Vanstraelen & Benková, 2012). Moreover, shoot branching may involves stigolactones as hormone that control plant branching development.

In WRKY8-1, the expression of RBOHD was higher under the control condition while RBOHF expression was slightly higher under salt treatment, but overall gene expression was very low. Regulation of these genes might be related to the regulation of hormonal pathways. In line with the low expression of the NADPH pathway, in WRKY8-1 the ethylene pathway was down-regulated under salt conditions. (Mersmann, Bourdais, Rietz, & Robatzek, 2010) reported that the oxidative burst was diminished in ethylene-insensitive mutants. Accumulation of Flagellin Sensitive2 (FLS2) transcripts was reduced in *etr1* and *ein2*, indicating a necessity for ethylene signaling in FLS2 expression. In overexpression WRKY8-1 better growth under salt condition was correlated with down regulation of ethylene pathway and might be related to down regulation of defense responses as NADPH pathway also down regulated under salt condition.

CONCLUSIONS

1. Most of the transgenes were showed better growth performance in control and salt treatments compared to the WT (MM) eg. WRKY7-1 and WRKY8-1.
2. Some of transgene showed change in phenotypic compare to WT. Most of the change of this phenotypic correlated with the level expression of transgene.
3. The phenotypic change in WRKY8-1 indicated pleiotropic effect.
4. Highest EL in WRKY8-1 indicated higher cell damage while lower EL in WRKY7-1 indicated the ability of plant to reduce the stress.
5. The higher accumulation of ions Na+ and Cl- in possibly related to the ability of this line in ion compartmentalization.
6. Most of overexpression lines had higher gene expression compared to wild type (MM). There were different level of gene expression among independent lines in the same WRKY gene.
7. Salt stress caused variations in the expression levels of genes encoding ROS scavenging, NADPH pathway, plasma membrane cell death, and hormonal pathways in line that reflected the pathway that may involve in response to salt stress.

RECOMMENDATIONS

1. Genotypes WRKY3, WRKY5, WRKY7, WRKY8 are recommended as genotypes of interest to study in future experiments.
2. More independent lines should be screened for each of the overexpressors to establish a better correlation between gene expression/ function and the phenotypic responses.
3. With some improvement in the technical aspects (eg. incubation in continuously treatment with high light intensity), paraquat treatment is a good method to evaluate sensitive or tolerant genotypes to oxidative stress conditions.
4. Additional analysis of the WRKY promoter region of WRKY7-1 and WRKY8-1 to obtain an overview of genetic regulation in the interaction of these WRKYs
5. PCR products of the primers targeting the WRKY genes should be sequenced to verify that they target the specific WRKY genes
6. A transcriptional profiling study by microarray might be very important to get a wider view of the gene regulation occurring in selected WRKY overexpression lines under control and salt treatments.
7. The compilation of observation data for the WRKY overexpression lines with WRKY RNAi (mutant) lines under salt condition could provide more complete information about genes that regulate plant responses to salinity stress.
8. Application of exogenous hormones in WRKY overexpression lines and WRKY RNAi lines could provide valuable information about the regulation of hormonal pathways during salt stress.

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APPENDIX

7.1. Growth Parameters

Table 5. Analysis of variance for growth traits (P value), genotype mean value, coefficient of variation (CV) of the absolute measurement under control and salt treatment

Traits	Control			Salt		
	P value	Mean	% CV	P value	Mean	% CV
Height Week 2	<.001	22.93	11	<.001	24.15	14.9
Height Week 3	<.001	51.2	11.4	<.001	42.41	10.5
Final Height	<.001	102.6	9.2	<.001	77.07	8.6
Number of Leaves	<.001	12.97	8.1	<.001	13.37	8.5
Fresh Weight	<.001	252	11.6	<.001	206.2	6.7
Dry Weight	<.001	21.62	15.7	<.001	28.7	8.6
Chlorophyll Content	0.016	42.96	5	<.001	52.57	6.2

Table 6. Genetic variation of absolute plant height and number of leaves under control and salt treatment

Genotypes	Control				Salt			
	Plant Height		Leaves Number		Plant Height		Leaves Number	
MM	98.5	cde	12.5	bcd	75.02	cde	9.37	a
WRKY1-1	107.3	defg	13	bcde	73.77	cd	12.5	b
WRKY1-2	108.7	defgh	12.33	bc	73.77	cd	12.5	b
WRKY1-3	96.7	cd	13	bcde	65.77	bc	13	bc
WRKY2-2	118.7	fghi	13.33	cdef	86.77	fg	13	bc
WRKY3-1	69	b	11.5	b	69.85	bcd	13.25	bcd
WRKY3-2	119.3	ghi	14.17	def	81.02	def	13.25	bcd
WRKY 3.3	88.3	c	13	bcde	63.02	b	13.5	bcd
WRKY4-1	108.3	defgh	13	bcde	86.27	f	13.5	bcd
WRKY4-2	123.3	hi	13	bcde	83.27	ef	13.75	bcd
WRKY4-3	112.7	efgh	12.67	bcde	86.92	fg	13.75	bcd
WRKY5-1	116.7	fgh	15	f	87.02	fg	14	bcd
WRKY7-1	132.3	i	14.33	ef	95.77	g	14.02	bcd
WRKY 7.3	109.3	defgh	14.17	def	85.02	f	14.12	cd
WRKY8-1	26.2	a	8.33	a	45.52	a	14.25	cd
WRKY9-2	103.5	cdef	13.5	cdef	75.02	cde	14.5	cd
WRKY9-3	105.7	defg	13.67	cdef	72.77	cd	14.75	d

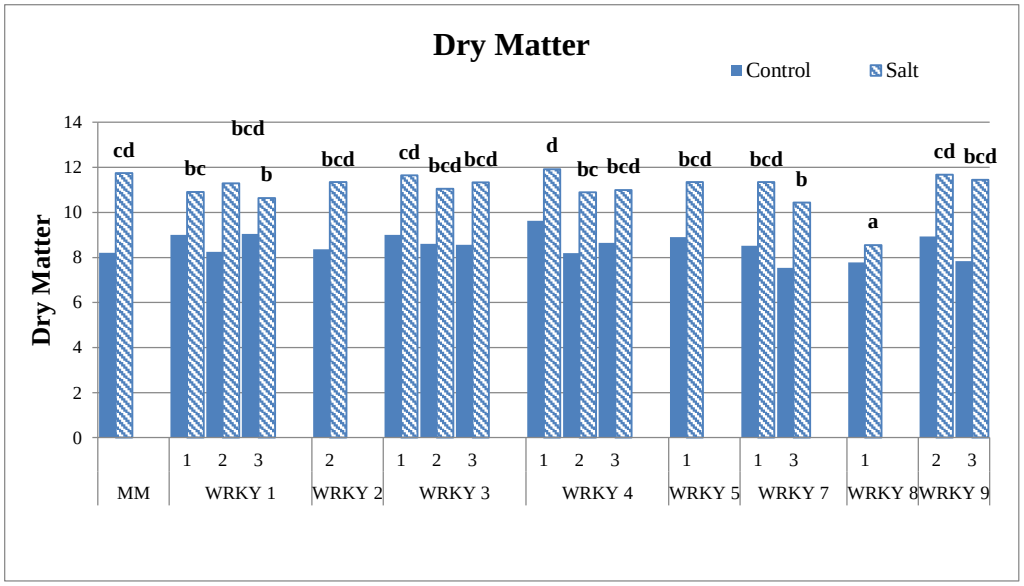


Figure 20. Effect of salt treatment (100mM NaCl) to dry matter of MM and WRKY overexpression lines. Different letters indicates significant difference at $P < 0.05$.

7.2. Electrolyte Leakage (%)

Table 7. Analysis of variance for percentage of EL at paraquat 0.5 µM and 1 5 µM treatment

Hours	Paraquat 0.5 µM			Paraquat 1 µM		
	P value	Mean	% CV	P value	Mean	% CV
24	0.003	31.88	19	<.001	52.12	16
72	0.008	43.67	16.8	<.001	69.15	16
48	<.001	70.98	11	<.001	93.92	9.1

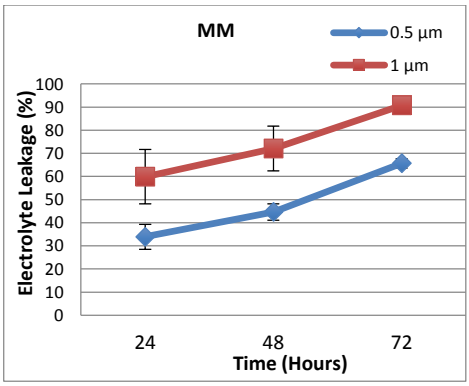
Table 8. Genetic variation of %EL under paraquat 0.5 µM treatment

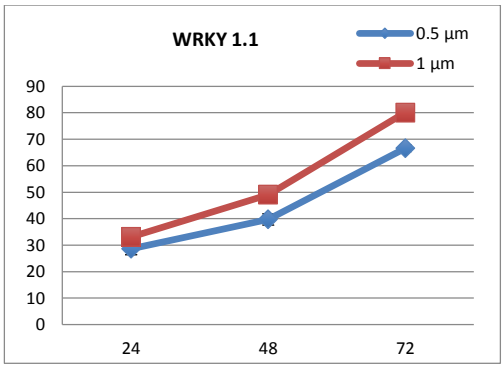
Genotypes	Paraquat 0.5 µM					
	24 hours		48 hours		72 hours	
MM	33.91	bcde	44.64	abcd	65.69	bcd
WRKY1-1	28.65	abcd	39.74	abcd	66.6	bcd
WRKY1-2	28.65	abcd	38.7	abc	60.28	b
WRKY1-3	34.26	bcde	48.87	cde	91.6	f
WRKY2-2	24.04	a	34.03	a	61.89	b
WRKY3-1	46.26	f	57.17	e	77.5	de
WRKY3-2	26.83	abcd	36.75	ab	59.12	b
WRKY3.3	28.18	abcd	37.25	abc	64.64	bc
WRKY4-1	34.44	cde	47.09	bcde	67.25	bcd
WRKY4-2	35.74	de	46.02	bcde	71.38	bcde

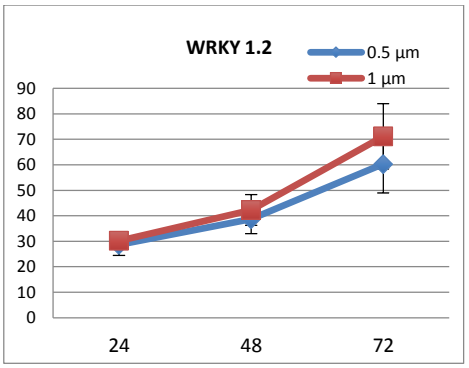
WRKY4-3	33.47	abcde	47.71	bcde	78.07	de
WRKY5-1	40.62	ef	50.72	de	74.98	cde
WRKY7-1	24.59	ab	37.41	abc	68.13	bcd
WRKY7.3	26.51	abcd	33.15	a	41.79	a
WRKY8-1	29.63	abcd	47.76	bcde	83.01	ef
WRKY9-2	27.85	abcd	37.98	abc	66.6	bcd
WRKY9-3	25.07	abc	36.65	ab	63.85	bc

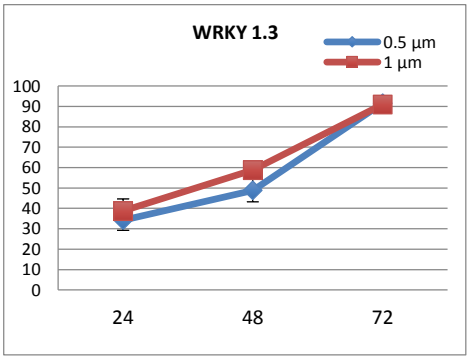
Table 9. Genetic variation of %EL under paraquat 1 µM treatment

Genotypes	Paraquat 1 µM					
	24 hours		48 hours		72 hours	
MM	59.91	efg	72.09	cdef	90.78	d
WRKY1-1	33.13	ab	49.09	ab	80.11	abcd
WRKY1-2	30.2	a	42.29	a	71.16	abc
WRKY1-3	38.7	abc	58.82	abcdef	91.03	d
WRKY2-2	51.87	cdef	68.44	cdef	88.63	d
WRKY3-1	69.32	g	73.05	defg	89.51	d
WRKY3-2	46.92	bcdef	55.79	abc	83.93	cd
WRKY3.3	61.42	fg	73.66	efg	89.7	d
WRKY4-1	56.61	defg	74.4	fg	92.58	d
WRKY4-2	44.97	abcde	56.53	abcd	81.91	bcd
WRKY4-3	38.75	abc	59.05	abcdef	85.12	d
WRKY5-1	41.19	abcd	57.22	abcde	79.84	abcd
WRKY7-1	39.32	abc	58.44	abcdef	67.98	a
WRKY7.3	33.04	ab	49.29	ab	69.44	ab
WRKY8-1	59.72	efg	89.32	g	107.34	e
WRKY9-2	61.05	fg	72.09	cdef	87.24	d
WRKY9-3	50.45	cdef	64.62	bcdef	90.1	d


MM
0.5 µm
1 µm
Electrolyte Leakage (%)
Time (Hours)
24
48
72


WRKY 1.1
0.5 µm
1 µm
24
48
72


WRKY 1.2
0.5 µm
1 µm
24
48
72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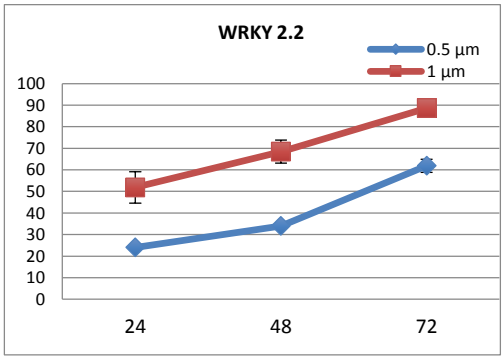
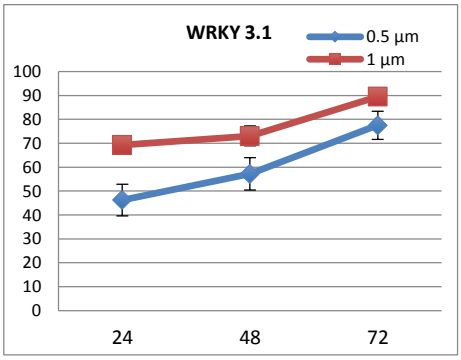
1 µm
24
48
72


WRKY 2.2
0.5 µm
1 µm
24
48
72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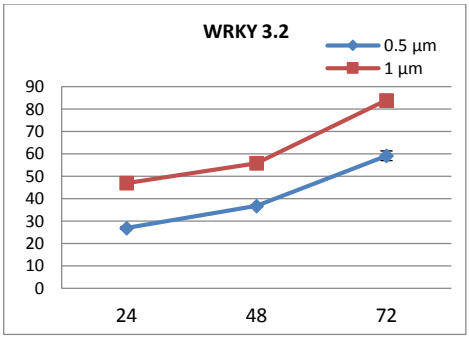
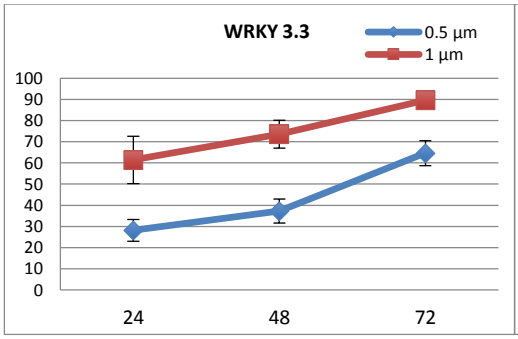
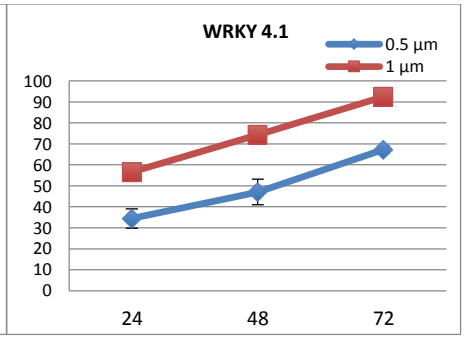
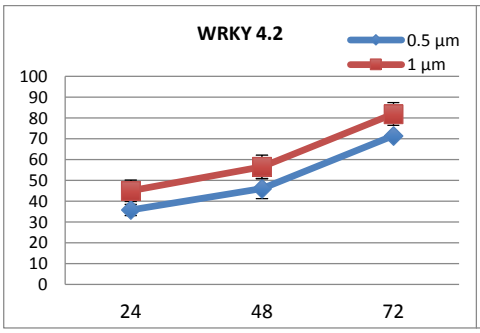
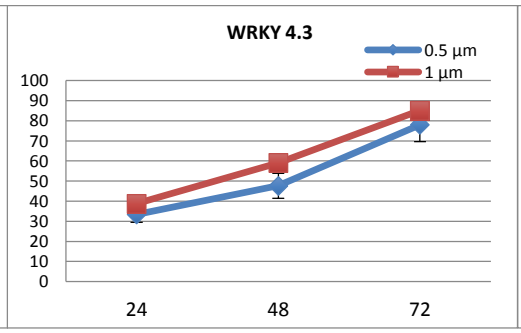
0.5 µm
1 µm
24
48
72

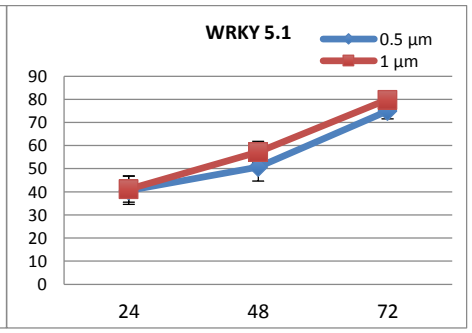

WRKY 3.2
0.5 µm
1 µm
24
48
72


WRKY 3.3
0.5 µm
1 µm
24
48
72


WRKY 4.1
0.5 µm
1 µm
24
48
72


WRKY 4.2
0.5 µm
1 µm
24
48
72


WRKY 4.3
0.5 µm
1 µm
24
48
72


WRKY 5.1
0.5 µm
1 µm
24
48
72

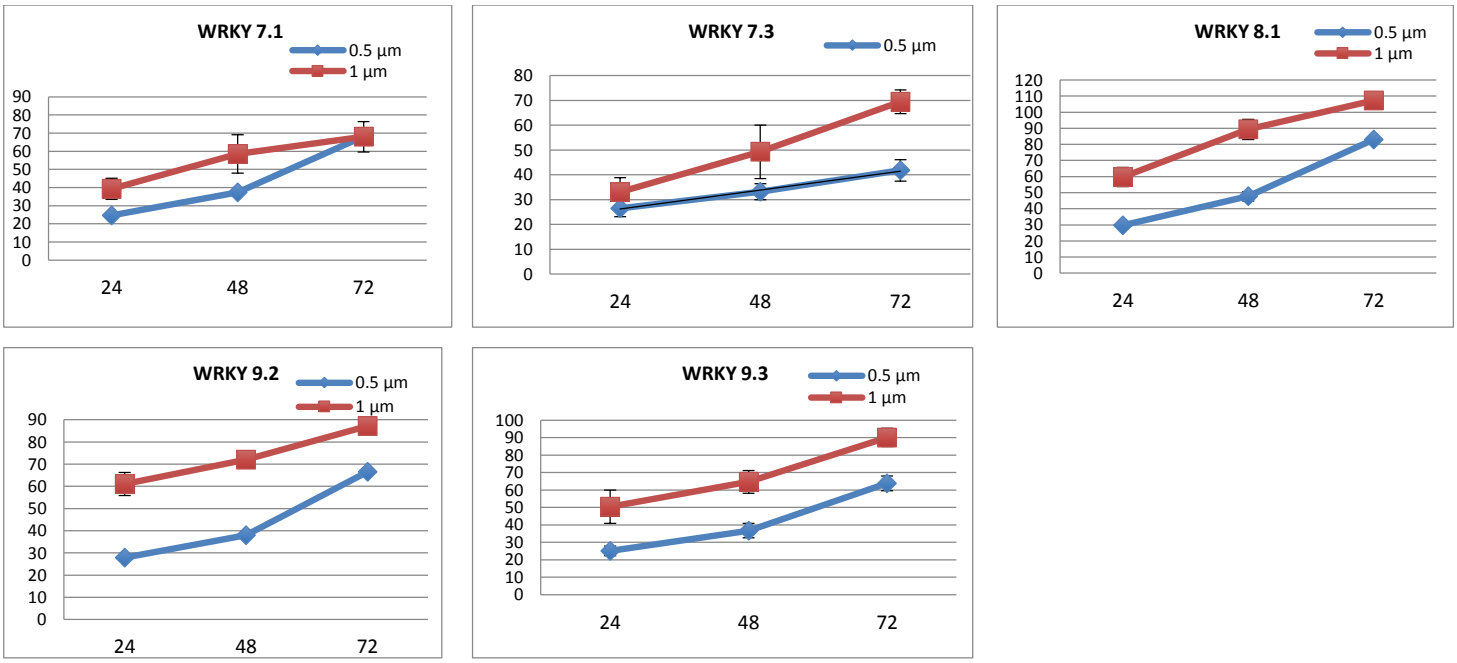


Figure 21. Percentage of EL of MM and WRKY overexpression lines at 24,48, and 72 hours time points under the treatmrnt of 0.5 µM and 1 µM paraquat. The bar represented standard error.

7.3. Ion Content Analysys

Table 10. Analysis of variance of ion content (mg/g) at control condition

Ion	Control					
	Leaves			Stem		
	P value	Mean (mg/g)	% CV	P value	Mean (mg/g)	% CV
Chloride	0.4	3.11	10.8	<.001	4.93	11.1
Phosphate	0.082	17.5	20.1	<.001	14.66	18.4
Sulfate	0.22	30.74	16	<.001	10.8	10.8
Potassium	0.003	50.06	12.4	0.69	71.08	12.6
Sodium	0.14	8.91	31.9	0.22	11.32	32.2
Magnesium	0.381	17.8	14.9	<.001	8.33	13.4
Calcium	0.714	14.3	16.7	<.001	4.35	17

Table 11. Analysis of variance of ion content (mg/g) at sat treatment

Ion	Salt					
	Leaves			Stem		
	P value	Mean (mg/g)	% CV	P value	Mean (mg/g)	% CV
Chloride	0.001	32.81	16.8	<.001	27.02	15.2
Phosphate	0.002	13.52	23.4	0.038	12.41	20.7
Sulfate	0.3	19.5	20.1	0.27	6.35	24.7
Potassium	0.007	30.61	19.3	0.002	35.7	20.7
Sodium	0.121	34.37	22.1	0.014	25.97	20.2
Magnesium	0.212	14.37	19.5	<.001	6.33	15.4
Calcium	0.023	13.66	18.2	0.005	4.27	17.8

Table 12. Genetic variation of ion content (mg/g) at control treatment

Treatment Control	Ion Content (mg/g)							
	Genotypes	Chloride	Phosphate	Sulfate	Potassium	Sodium	Magnesium	Calcium
Leaves	MM	3.153 a	17.01 a	36.05 a	52.84 cde	8.722 a	17.79 a	15.17 a
	WRKY1-1	3.012 a	15.4 a	28.61 a	45.67 abc	10.304 a	15.92 a	12.89 a
	WRKY1-2	3.081 a	15.78 a	26.41 a	47.93 bcd	8.187 a	13.17 a	11.53 a
	WRKY1-3	3.012 a	18.35 a	26.53 a	36.93 a	9.361 a	16.64 a	13.95 a
	WRKY2-2	3.326 a	18.31 a	29.35 a	54.9 cde	6.331 a	17.79 a	14.45 a
	WRKY3-1	2.96 a	17.86 a	28.11 a	45.38 abc	10.354 a	18.42 a	16.1 a
	WRKY3-2	3.101 a	15.66 a	30.67 a	48.36 bcd	8.299 a	15.92 a	12.86 a
	WRKY3.3	3.015 a	19.1 a	34.41 a	55.98 de	8.992 a	18.71 a	14.46 a
	WRKY4-1	2.771 a	15.04 a	35.63 a	42.18 ab	12.633 a	16.87 a	14.65 a
	WRKY4-2	3.327 a	18.01 a	32.61 a	56.19 de	8.85 a	17.97 a	14.56 a
	WRKY4-3	3.057 a	16.7 a	34.95 a	55.59 cde	6.532 a	17.63 a	14.73 a
	WRKY5-1	2.969 a	14.09 a	30.35 a	45.59 abc	11.287 a	16.63 a	13.79 a
	WRKY7-1	2.926 a	15.41 a	31.41 a	49.32 bcd	8.027 a	15.98 a	13.3 a
	WRKY7.3	3.605 a	18.06 a	30.81 a	54.87 cde	12.359 a	17.43 a	16.59 a
	WRKY8-1	3.06 a	24.88 a	19.27 a	49.58 bcd	4.777 a	20.51 a	14.57 a
	WRKY9-2	3.096 a	13.1 a	31.3 a	47.04 abcd	7.852 a	16.75 a	14.47 a
	WRKY9-3	3.412 a	18.79 a	36.08 a	62.69 e	8.729 a	18.01 a	14.97 a
Stem	MM	4.721 abc	14.07 abc	10.81 abcdef	79.3 a	11.58 abc	7.572 abc	4.064 abc
	WRKY1-1	4.091 a	13.28 abc	9.77 abcd	65.69 a	10.21 abc	6.756 ab	3.665 ab
	WRKY1-2	4.715 abc	13.79 abc	10.5 abcdef	68.19 a	10.69 abc	6.83 ab	3.52 a
	WRKY1-3	5.429 cd	14.04 abc	10.2 abcde	72.22 a	15.39 c	10.469 ef	4.874 bcd
	WRKY2-2	5.053 bcd	15.71 bc	10.98 bcdef	72.11 a	12.26 abc	9.255 cde	4.643 abcd
	WRKY3-1	5.759 d	16.91 c	13.24 g	66.57 a	15.38 c	10.551 ef	5.693 d
	WRKY3-2	4.546 abc	14.89 abc	11.91 efg	72.03 a	10.95 abc	7.664 abc	4.067 abc

	WRKY3.3	5.434	cd	14.06	abc	12.32	fg	78.91	a	8.87	ab	9.913	de	3.975	abc
	WRKY4-1	4.375	ab	12.01	ab	9.01	a	68.67	a	9.36	abc	7.027	ab	3.876	abc
	WRKY4-2	4.776	abc	13.58	abc	9.87	abcd	72.78	a	13.56	bc	7.52	abc	3.872	abc
	WRKY4-3	4.226	ab	10.91	a	9.27	abc	67.88	a	7.63	ab	6.587	a	3.438	a
	WRKY5-1	4.191	ab	13.44	abc	9.18	ab	70.33	a	8.53	ab	6.679	a	3.442	a
	WRKY7-1	4.226	ab	14.61	abc	11.68	defg	67.42	a	11.89	abc	7.224	ab	3.893	abc
	WRKY7.3	5.006	bcd	13.06	abc	11.13	cdef	70.81	a	13.43	bc	10.005	def	5.014	cd
	WRKY8-1	7.363	e	24.96	d	9.49	abc	69.41	a	7.26	a	11.85	f	7.394	e
	WRKY9-2	5.022	bcd	14.95	abc	11.93	efg	66	a	13.15	abc	7.087	ab	3.866	abc
	WRKY9-3	4.821	abc	14.9	abc	12.29	fg	80.03	a	12.32	abc	8.592	bcd	4.668	abcd

Table 13. Genetic variation of ion content (mg/g) at salt treatment

Treatment Salt	**Ion Content (mg/g)**														
	Genotypes	**Chloride**		**Phosphate**		**Sulfate**		**Potassium**		**Sodium**		**Magnesium**		**Calcium**	
Leaves	MM	31.96	abcd	11.85	a	17.4	c	31.35	abcde	29.8	a	13.61	a	12.6	abc
	WRKY1-1	31.28	abcd	12.77	ab	17.8	bc	27.21	abc	33.41	a	13.24	a	12.59	abc
	WRKY1-2	35.04	bcd	13.46	ab	20.5	ab	35.7	de	38.64	a	15.75	a	13.84	abc
	WRKY1-3	32.3	abcd	12.32	a	16.9	ab	26.52	ab	30.85	a	13.7	a	13.94	abc
	WRKY2-2	28.7	abc	11.23	a	16	bc	25.74	a	29.7	a	11.59	a	10.71	a
	WRKY3-1	34.6	bcd	13.39	ab	21	bc	30.24	abcd	35.15	a	16.33	a	13.69	abc
	WRKY3-2	38.47	d	14.8	ab	24.1	bc	39	e	42.61	a	16.64	a	15.23	c
	WRKY3.3	27.34	ab	11.29	a	17.7	bc	26.02	a	28.58	a	11.68	a	11.18	ab
	WRKY4-1	25.16	a	11.46	a	18.7	c	24.42	a	29	a	13.32	a	12.56	abc
	WRKY4-2	35.67	cd	16.84	b	22.3	bc	35.53	cde	37.34	a	15.04	a	14.42	bc
	WRKY4-3	28.38	abc	12.9	ab	19.4	c	26.21	a	32.76	a	13.42	a	13.08	abc
	WRKY5-1	30.15	abc	13.43	ab	21.3	bc	28.82	abcd	33.94	a	13.96	a	14.61	bc
	WRKY7-1	31.52	abcd	11.68	a	18.5	bc	29.02	abcd	31.69	a	13.75	a	12.57	abc
	WRKY7.3	35.08	bcd	13.18	ab	18.5	bc	34.84	bcde	36.51	a	14.74	a	13.61	abc

	WRKY8-1	46.41	e	22.75	c	18.9	a	27.28	abc	45.68	a	17.23	a	18.93	d
	WRKY9-2	32.23	abcd	13.08	ab	21.2	bc	36.95	de	33.35	a	14.34	a	14.33	bc
	WRKY9-3	33.5	bcd	13.49	ab	21.1	c	35.6	cde	35.35	a	15.96	a	14.3	bc
Stem	MM	23.44	abc	11.29	abc	5.64	a	34.51	abc	20.81	a	5.801	ab	3.998	abc
	WRKY1-1	27.02	bcd	13.82	bcde	6.56	a	39.04	bcd	25.58	ab	6.285	abcd	4.093	abc
	WRKY1-2	24.05	abc	12.87	abcde	5.75	a	32.76	abc	26.01	abc	5.825	ab	3.721	abc
	WRKY1-3	32.76	de	12.83	abcde	6.94	a	37.63	abcd	29.13	bcd	7.375	cd	4.712	cde
	WRKY2-2	23.9	abc	14.03	bcde	5.96	a	35.76	abcd	22.61	ab	5.614	ab	3.5	a
	WRKY3-1	33.74	e	12.22	abcd	7.8	a	33.32	abc	33.47	cd	9.821	e	5.744	e
	WRKY3-2	24.67	abc	11.85	abcd	5.84	a	29.78	abc	29.53	bcd	6.308	abcd	4.433	abcd
	WRKY3.3	26.8	abc	10.85	ab	8.63	a	30.8	abc	26.67	abc	6.291	abcd	4.449	abcd
	WRKY4-1	21.1	a	11.48	abcd	5.75	a	33.6	abc	23.95	ab	4.971	a	3.806	abc
	WRKY4-2	28.2	cde	14.84	cde	6.9	a	45.42	de	28.05	abcd	6.387	bcd	4.659	bcd
	WRKY4-3	26.36	abc	11.63	abcd	6.11	a	39.43	cd	26.47	abc	6.137	abc	3.959	abc
	WRKY5-1	24.08	abc	15.02	de	6.12	a	35.29	abcd	25.15	ab	6.335	abcd	4.435	abcd
	WRKY7-1	23.21	abc	11.44	abcd	5.95	a	27.54	a	23.74	ab	6.078	abc	4.387	abcd
	WRKY7.3	26.76	abc	10.13	a	5.33	a	33.18	abc	23.39	ab	6.52	bcd	3.794	abc
	WRKY8-1	49.14	f	15.87	e	6.92	a	53.2	e	34.33	d	7.624	d	5.42	de
	WRKY9-2	22.44	abc	11.31	abc	6.37	a	36.94	abcd	21.24	a	4.994	a	3.878	abc
	WRKY9-3	21.72	ab	9.46	a	5.34	a	28.77	ab	21.38	a	5.241	ab	3.62	ab

7.4. Gene Expression

Table 14. Absolute gene expression at control condition

WRKY Ox Lines	Mean Gene Expression	SE (Mean Gene Expression)	MM Gene expression	Gene Expression Relative to MM	SE (Gene Expression Relative to MM)
WRKY1-1	0.011118	0.001104	0.032416	0.342973	0.034044
WRKY1-2	0.018418	0.003967	0.032416	0.568185	0.122377
WRKY1-3	0.208128	0.001028	0.032416	6.420559	0.031721
WRKY2-2	0.001940	0.000463	0.000966	2.006978	0.479149
WRKY3-1	0.086198	0.006103	0.002247	38.353814	2.715689
WRKY3-2	0.001759	0.000010	0.002247	0.782880	0.004423
WRKY3.3	0.065338	0.011115	0.002247	29.071999	4.945740
WRKY4-1	0.003534	0.000848	0.002387	1.480535	0.355138
WRKY4-2	0.003382	0.001081	0.002387	1.416807	0.452864
WRKY4-3	0.001527	0.000086	0.002387	0.639682	0.035900
WRKY5-1	0.010285	0.005105	0.001257	8.184183	4.062307
WRKY7-1	0.125221	0.011629	0.036934	3.390439	0.314855
WRKY7.3	0.143037	0.014548	0.036934	3.872798	0.393898
WRKY8-1	0.147835	0.026364	0.001252	118.106135	21.062640
WRKY9-2	0.012177	0.000719	0.000039	314.105413	18.547479
WRKY9-3	0.011455	0.001728	0.000039	295.493965	44.586850