

REGULATION OF AUXIN RECEPTOR GENE FAMILY BY HORMONAL AND
ABIOTIC STRESS

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REGULATION OF AUXIN RECEPTOR GENE FAMILY BY HORMONAL AND
ABIOTIC STRESS

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ABSTRACT

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The plant hormone auxin controls growth and development by regulating the expression of many auxin responsive genes. TIR1/AFBs, a family of F-Box proteins act as receptors for auxin. In response to auxin, a group of transcriptional repressor proteins known as Aux/IAs are degraded through the ubiquitin-proteasome pathway involving SCF^{TIR1/AFBs}. According to recent studies, adaptive response to abiotic and biotic stresses is partly achieved through auxin signaling. It is also known that plant responses to salinity and osmotic stresses are partly controlled through abscisic acid (ABA) signaling, and ABA synthesis is enhanced in response to stress. Auxin signaling is also modulated

by ABA. Therefore, I sought to determine the effect of salinity and osmotic stress on the expression of auxin receptor F-Box genes. Furthermore, I examined the effect of ABA on auxin receptor gene expression. Finally, since gibberellic acid (GA) antagonizes ABA, I tested GA effects on auxin receptor genes. Our data indicate that *TIR1* expression is up-regulated in response to mild salinity, osmotic stresses, ABA and GA. Even though *TIR1*/*AFBs* are known to have similar functions, each of them is regulated differently by above conditions. The complex regulation of *TIR1*/*AFBs* may modulate the auxin response, and thereby adapt the plant to the changing environment. Molecular and physiological data support the hypothesis that modulation of auxin response through the regulation of auxin receptor genes leads to changes in physiological responses that ultimately may help the plant to adjust to adverse environments. Better understanding of molecular mechanisms involved in plant auxin response during environmental stress will enable scientists to develop superior crop plants that can thrive under adverse conditions. Further, understanding plant hormone crosstalk will fill the gaps in plant hormone signaling network.

CHAPTER I

INTRODUCTION

Plant growth and development is regulated by hormones and environmental factors. Arguably the major growth hormone, auxin was discovered many decades ago. Since then many other hormones such as abscisic acid (ABA), brassinosteroid, cytokinin, ethylene, gibberellins (GA), jasmonic acid, salicylic acid, and very recently strigolactone have been discovered (Chapman et al., 2009). Indole-3-acetic acid (IAA), the major natural auxin, controls many aspects of growth and development such as embryogenesis, apical dominance, flower development, lateral root initiation, phototropism, and gravitropism (Prasad et al., 1993, Marchant et al., 1999, Casimiro et al., 2001, Cheng & Zhao., 2007). Auxin stimulates the transcription of primary auxin responsive genes that fall in to three classes, *Aux/IAA*, *GH3* and *SAUR* (small auxin up RNA). The *Aux/IAA* proteins act as transcriptional repressors by binding to transcription factors called auxin response factors (ARF). There are 29 *Aux/IAA* genes and 23 *ARFs* in the *Arabidopsis* genome (Chapman et al., 2009). The transport inhibitor response 1 (TIR1) and *Aux/IAA* proteins act as co-receptors for auxin (Tan et al., 2007). TIR1 is an F-box protein that

belongs to a family of F-box proteins known as auxin signaling F-box proteins (AFBs). Three other F-Box proteins (AFB1, 2 and 3) in this family have also been found to function as co-receptors for auxins (Dharmasiri et al., 2005a). These F-box family proteins contain leucine rich repeats (LRR) that are involved in interacting with auxin and Aux/IAA proteins (Dharmasiri et al., 2005b). It has been shown that the bottom of a single top surface pocket in the TIR1-LRR domain binds with auxin. Aux/IAA proteins are docked to the upper part of the surface pocket immediately above the auxin binding site and completely cover the auxin molecule. Auxin acts as a molecular glue to attach Aux/IAA to the TIR1/AFBs (Tan et al., 2007). TIR1/AFB F-box proteins are part of a protein complex called E3 ubiquitin ligases that contain three other proteins called Skp1, Cullin1 and Rbx1, and are thus known as SCF complex. After binding to SCF^{TIR1/AFB} complex in the presence of auxin, Aux/IAA undergoes ubiquitination and subsequent degradation via 26S proteasome pathway (Gray et al., 2001). Release of repression due to Aux/IAA degradation leads to the activation of gene transcription by ARFs (Dharmasiri et al., 2004).

There are about 700 genes that possibly encode F-box proteins in the *Arabidopsis* genome. Phylogenetic analysis reveals that they can be divided into 5 families and 20 sub-families, suggesting their vast diversity. Auxin receptor genes belong to the C3 sub-family. All of these F-box proteins contain a conserved F-box domain with 60 amino acids that interacts with Skp1 of the SCF complex (Gagne et al., 2002). *TIR1*, *AFB1*, *AFB2* and *AFB3* genes express in overlapping regions of *Arabidopsis* seedlings and act in a redundant manner (Dharmasiri et al., 2005b, Parry et al., 2009). The stepwise introduction of the four mutant F-box auxin receptor genes causes progressive decrease in

Arabidopsis auxin response with increasingly severe defects in development (Dharmasiri et al., 2005b). Due to sequence similarity, expression pattern and activity, *TIR1* and *AFB1* are considered paralogs. *AFB2* and *AFB3* are closely related to each other but distantly related to *TIR1* and *AFB1* (Dharmasiri et al., 2005b). However, according to Parry et al., (2009) *TIR1* makes the major contribution to auxin signaling in roots, followed by *AFB2*. The contribution of *AFB1* and *AFB3* is evident only in higher order mutants, suggesting their minor contribution to the auxin response. Neither *AFB1* nor *AFB2* rescue the *tir1-1* auxin resistant phenotype, suggesting the distinct nature of these proteins in spite of their similarity (Parry et al., 2009).

Expression levels of auxin receptor F-box genes are known to be regulated by microRNAs (Sunkar et al., 2004, Navarro et al., 2006, Vidal et al., 2009, Ammour et al., 2011). MicroRNAs (miRNAs) are a class of small, non-coding, single stranded RNA molecules approximately 21-25 nucleotides in length. They are well characterized for the down-regulation of gene expression either by degradation of mRNAs or repressing translation (reviewed by Lin et al., 2009). *miR393* has been identified as a stress inducible miRNA that targets F-box auxin receptor genes. It is derived from either *miR393a* or *miR393b* genes. *miR393* is induced by cold, dehydration, salinity, ABA (Sunkar et al., 2004) and microbes (Navarro et al., 2006). Bacterially derived flagellin 22 induces expression of *miR393* from the *miR393a* gene. *miR393* modulates expression of all the known auxin receptor genes, except for *AFB1* (Navarro et al., 2006). According to Vidal et al., (2009) nitrate induces *miR393* in roots, effecting down-regulation of *AFB3* without affecting the other auxin receptors. Interestingly, a recent study by Ammour et al., (2011) showed that in developing leaves expression of all four auxin receptor genes is

down-regulated by *miR393* derived mainly from *miR393b*. Additionally, there is evidence for the generation of small interfering RNAs (siRNAs) mainly from the transcripts of *AFB2* and *AFB3* with the aid of *miR393*. These siRNAs are proposed to regulate auxin receptor F-box genes as well as downstream auxin responsive genes (Ammour et al., 2011). Therefore, the regulation of the expression of F-box auxin receptor genes by *miR393* involves a complex mechanism in which the origin of *miR393* and the auxin receptor transcripts it targets are regulated by environmental cues.

Plants have an enormous plasticity for adaptation to environmental challenges. In this process plant hormone ABA plays a major role in adapting the plants to such environments (Rohde et al., 2000). Drought, high salinity, low temperature and pathogen attacks increase the level of ABA synthesis, leading to changes in gene expression and subsequent physiological changes in plants (Dallaire et al., 1993). ABA is involved not only in stress responses but also in regulating leaf size, inter-node length, seed dormancy, bud dormancy, embryo and seed development, and reproduction (Rock et al., 2000).

The recent discovery of RCAR/PYR1/PYL as ABA receptors has given more insight into ABA signaling pathways in plants (Hao et al., 2010). The promoter regions of ABA responsive genes contain conserved *cis*-regulatory sequences (c/tACGTGGC) called ABA responsive elements (ABREs). The ABA specific transcription factors known as ABA responsive element binding factors (ABFs) or ABA responsive element binding proteins (AREBs) bind to ABREs and regulate ABA dependent gene transcription. ACGT residues of ABREs act as the core motif for the binding of ABFs (Hattory et al., 2002). ABFs (or AREBs) are a sub-family of bZIP transcription factors (Raghavendra et al., 2010). The major transcription factors in this sub-family such as

ABF1, ABF2, ABF4 and ABI5 are phosphorylated prior to activating transcription (Fujii et al., 2009). SnRKs (Sucrose non-fermenting related kinases) and OST1 (Open stomata 1) are kinases that are responsible for phosphorylating these ABFs (Sirichandra et al., 2010). In the absence of ABA, these kinases are dephosphorylated by type 2C protein phosphatases (PP2Cs), blocking transcription. Binding of ABA to RCAR/PYR1/PYL deactivates PP2Cs, thereby enhancing the phosphorylation of transcription factors (ABFs) and the transcription of ABA responsive genes (Raghavendra et al., 2010). Additionally, there are other kinases such as calcium dependent protein kinases (CDPKs) that are capable of phosphorylating ABA specific transcription factors (Zhu et al., 2007). In addition to ABFs, other transcription factors such as MYC and MYB are also involved in ABA induced gene transcription (Abe et al., 2003).

Gibberellin (GA) is another phytohormone that controls many aspects of plant development including seed germination, leaf expansion, stem elongation, flowering, and seed development (Gubler et al., 2004). The synthesis of active form of GAs that are tetracyclic diterpenoids such as GA₁, GA₄ and GA₅ from geranylgeranyl diphosphate has been studied in depth (Ogava et al., 2003). GA receptor gibberellin insensitive dwarf 1 (GID1), a soluble protein localized to both cytoplasm and nucleus, perceives the GA signal (Sun., 2010). A group of proteins called DELLA proteins act as transcriptional repressors of GA responsive genes. There are five DELLA proteins in *Arabidopsis* known as GAI, RGA, RGL1, RGL2, and RGL3 (Olszewski et al., 2002). Interaction between GID1 and DELLA proteins is enhanced by the binding of GA to GID1, resulting in rapid degradation of DELLAs via the ubiquitin proteasome pathway (Sasaki et al., 2003). A specific ubiquitin E3 ligase complex (SCF^{SLY1/GID2}) is required to recruit

DELLA proteins for polyubiquitination and subsequent degradation by the 26S proteasome (reviewed by Sun, 2010). Transcription of GA responsive genes is also regulated by binding of transcription factors to regulatory elements. The Myb family of transcription factors is known to interact with GA response complex (Gubler et al., 1992). In this case, the core GARE (gibberellic acid responsive element) sequence TAACAAA acts along with other elements such as pyrimidine box (C/TCTTTT), TATCCAC box and CAACTC box to form a GA response complex (Rogers et al., 1992) to regulate GA dependent transcription.

A number of physiological studies have indicated that significant crosstalk exists among plant hormones such as auxin, ABA, GA, cytokinin and ethylene (Gazzarrini et al., 2003). It is known that auxin and GA can affect each other's biosynthesis in a positive manner (Ogawa et al., 2003). Polar auxin transport is important for DELLA mediated GA responses (Kanyuka et al., 2003). GA and ABA show antagonistic effects on seed germination (Piskurewicz et al., 2008). GA enhances the proteasome-mediated destruction of a key DELLA factor that represses germination, thereby promoting the seed germination. Conversely, ABA blocks germination by inducing a transcription factor that represses germination (Ogawa et al., 2003). Further, negative regulation of auxin response factor 10 (*ARF10*) by *miR160* plays a critical role in seed germination and post-embryonic development through auxin-ABA crosstalk (Liu et al., 2007). Interestingly, many GA-regulated genes contain both GA- and ABA-responsive promoter elements, suggesting fine tuning of the transcription of these genes by both hormones (Busk et al., 1998). ABA modulates auxin response under different environmental conditions. Especially, it affects auxin biosynthesis and transport. For example, in

response to salinity stress ABA level is enhanced altering the expression of auxin transporter genes (Yu et al., 2010). Additionally, ABA induces expression of *miR393* (Sunker et al., 2004) which targets auxin receptor family transcripts for degradation.

Environmental cues such as salinity and osmotic stress affect plant hormone signaling pathways including auxin and ABA signaling. Salt and osmotic stresses are a major concern in agriculture. These environmental stresses cause serious damages to crop plants, thereby causing staggering economic losses (Zhu et al., 2001). These stress conditions affect many processes in plants such as ion and osmotic homeostasis, cell division and expansion, and regulation of detoxification mechanisms (Zhu., 2002). Therefore, many signaling pathways are activated during stress in order to overcome these challenges. Accumulation of ABA in response to osmotic stress as a result of both enhancement of ABA synthesis and inhibition of ABA degradation has been demonstrated (Koornneef et al., 1998). There are ABA dependent and ABA independent pathways involved in osmotic stress tolerance (Shinozaki et al., 1997). During ABA dependent osmotic stress response, transcription factors such as ABFs/AREBs, MYCs and MYBs bind to cis-regulatory elements of target genes and induce transcription (Huang et al., 2011). Genes that are induced by osmotic stress, but independent of ABA signaling, contain cis-regulatory elements called dehydrate responsive elements (DRE) (Shinozaki et al., 1994). A family of proteins called DRE binding (DREB) proteins interacts with DREs and regulates transcription of osmotic stress induced genes (Nakashima et al., 1999).

The salt overly sensitive (SOS) pathway is a prominent signaling pathway involved in ion homeostasis during salinity stress. Salinity stress also induces ABA

synthesis and ABA signaling in plants independent of osmotic effects (Wang et al., 2001, Guo et al., 2011). Normal auxin distribution in the root is affected by salinity stress, resulting in altered lateral root development (Sun et al., 2008). The auxin signaling cascade is also affected by salinity. Different F-box receptor genes are regulated differently under salinity stress, suggesting that auxin signaling is modulated in response to salinity stress (Iglesias et al., 2010). According to Wang et al., (2009) salinity alters auxin distribution in roots and thereby abolishes lateral root initiation, and promotes lateral root elongation. Therefore, it is likely that the outcome of adapted response to salinity and osmotic stress is a combined effect of auxin, ABA as well as other signaling pathways.

Although the modulation of auxin signaling by different environmental cues and other plant hormones has been known for some time, the underlying molecular mechanisms have not been elucidated. One way of regulating a signaling cascade is changing the abundance of proteins involved. Hence, hypothesizing that changes in the abundance of auxin receptor family proteins consequent to regulating transcription, translation or protein stability may affect downstream signaling pathways, I focused on the regulation of the auxin receptor F-box gene family in response to salinity stress, osmotic stress, abscisic acid and gibberellic acid. To understand gene expression, transcriptional as well as translational gene constructs fused to the reporter gene β -glucuronidase (GUS) were used. In order to confirm the reporter gene expression data, RT-PCR and physiological assays were carried out. To check the *miR393* expression, transcriptional gene constructs fused to reporter gene green fluorescent protein (GFP) were used. Bioinformatics data suggested the presence of putative *cis*-regulatory

elements associated with stress and hormonal responses in auxin receptor genes. The function of one such putative *cis*-regulatory element, ABRE in auxin signaling was studied by site directed mutagenesis.

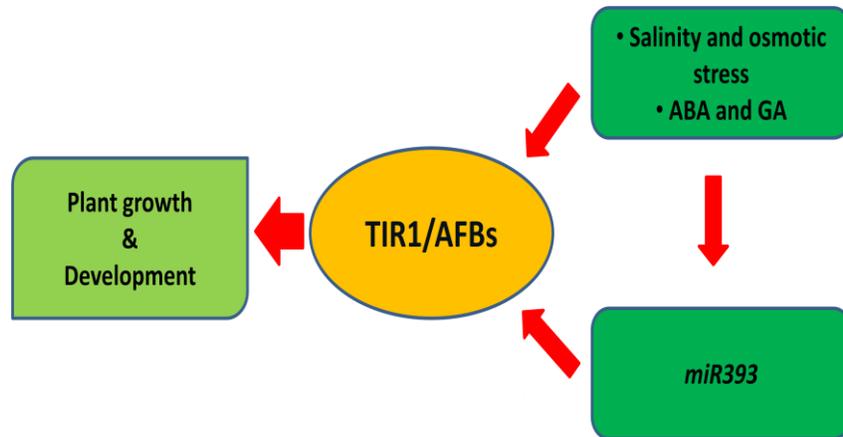


Figure 1. Schematic diagram of possible pathways to regulate the abundance of TIR1/AFBs. Expression of auxin receptor F-box genes may be directly regulated by environmental factors such as salinity and osmotic stress as well as ABA and GA. The above conditions may also modulate miR393 expression, in turn regulating auxin receptor gene expression. The abundance of TIR1/AFBs may ultimately affect plant growth and development through auxin signaling pathway.

CHAPTER II

MATERIALS AND METHODS

Plant varieties and growth conditions

Arabidopsis thaliana (L.) Heynh. Var. *Columbia* (Col-0) and *Wassilewskija* seeds obtained from the Arabidopsis Biological Resource Center, Ohio State University, were used as wild type in all experiments performed. *TIR1::GUS*, *TIR1::TIR1-GUS*, *AFB1::GUS*, *AFB1::AFB1-GUS*, *AFB2::GUS*, *AFB2::AFB2-GUS*, *AFB3::GUS*, *AFB3::AFB3-GUS* reporter lines, *tir1-1*, *tir1-9*, *afb1-1*, *afb2-1*, *afb3-1*, *afb2-1/afb3-1*, *afb1-1/afb3-1*, *tir1-1/afb1-1*, *tir1-1/afb2-5*, *tir1-1/afb5-6*, *afb1-3/afb2-5*, *tir1-1/afb2-5/afb3-4*, *afb1-1/afb2-1/afb3-1*, and quadruple mutant lines, as well as *GVG::TIR1-Myc* and *35S::AFB1-Myc* over expression lines were provided by Dr. Mark Estelle. *miR393a::GFP* and *miR393b::GFP* seeds were provided by Dr. Lionel Navarro.

Seeds were surface sterilized with 40% bleach with 0.1% TritonX-100 and thoroughly rinsed with sterile distilled water. Seeds were plated on either *Arabidopsis thaliana* medium with 1% sucrose (ATS), pH 5.6 (Lincoln et al., 1990), or where specified on 0.5X Murashige and Skoog medium (MS; Murashige and Skoog, 1962; Sigma) with 1% sucrose, pH 5.6. The plates were incubated at 4°C for 24 hours and then transferred to a growth chamber at 22°C with continuous illumination (Dharmasiri et al., 2003). All experiments on sterile medium were performed in the same growth chamber.

Treatments for reporter gene expression

To test the effect of NaCl and mannitol on expression of auxin receptor family genes, seedlings carrying transcriptional and translational GUS reporter constructs were used. Seedlings were grown as mentioned above for four days. They were transferred to ATS liquid media containing 100-300 mM NaCl or mannitol, 50-200 μ M ABA or 50-200 μ M GA. Seedlings were incubated for 18 hrs following treatment under continuous light and gentle agitation. All the treatments were carried out in 24 well microtiter plates.

Histochemical staining

Histochemical staining of seedlings for GUS assays was carried out according to Jefferson et al. (1987). Briefly, treated seedlings were washed with distilled water and fixed using GUS fixer (0.3 M mannitol, 10 mM 4-morpholineethanesulfonic acid (MES), 0.3% formaldehyde) for 40 minutes with gentle shaking. They were washed three times, five minutes each with 100 mM phosphate buffer (pH 7.0). Fixed seedlings were incubated with GUS staining buffer (0.1 M 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium, 100 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide) until color developed after vacuum infiltration.

Quantitative β -glucuronidase assay

For quantitative β -glucuronidase assays seedlings were frozen in liquid nitrogen immediately after treatments. All the tissues were homogenized in GUS extraction buffer (100 mM phosphate buffer, pH 7.0, EDTA, 0.1% sodium lauryl sarcosine, 10 μ M β -mercaptoethanol). Supernatant was collected after centrifugation at 10,000 X g for 10

minutes. Amount of total protein was quantified using Bradford assay (Bradford, 1976). Equal amounts (60-70 μg) of total protein from each treatment were incubated up to 1 hour in the assay buffer containing 4-methylumbelliferyl- β -D-glucuronide hydrate (4-MUG) in the extraction buffer. The reaction was stopped by adding 0.2 M sodium carbonate. Fluorescence was measured at a wavelength of 460 nM using a luminometer (Turner, Sunnyvale, CA, Model number-9200-002). All the experiments were carried out in triplicate.

Germination assays

Seeds were surface sterilized as described above and plated on ATS media containing NaCl, mannitol, ABA or GA. Plates were vernalized at 4°C for 48 hours and transferred in to a growth chamber at 22°C with continuous illumination. Seedlings were grown for 6 to 7 days. Number of total seedlings and the seedlings having green cotyledons were counted, and percentage of green cotyledons was calculated. All the experiments were repeated at least three times.

RNA isolation and RT PCR

Arabidopsis thaliana (L.) Heynh. Var. *Columbia* (Col-0) wild type seedlings were grown for 4 days in ATS media and then treated with NaCl, mannitol, ABA and GA for 18 hours as mentioned above. Seedlings were then frozen in liquid nitrogen and stored at -80°C until further use. For RNA extraction, frozen tissues were ground in liquid nitrogen to a fine powder. Total RNA was extracted using TriReagent (Sigma, St.Louis, MO) according to the manufacturer's instructions. RNase-free DNase was used to remove any

contaminating DNA from the extract. cDNA was synthesized using total RNA and Superscript reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The amount of RNA in each preparation was standardized by the PCR amplification of ubiquitin cDNA and quantification of band intensity using ImageJ software. cDNA solutions that contained the same amount of RNA were used for the PCR amplification of auxin receptor genes.

Identification of regulatory sequences

The promoter sequences of auxin receptor gene family were analyzed using the following bioinformatics software, ATHAMAP (Institute of Genetics at the Technical University of Braunschweig, Germany), ATHENA (Washington State University, Washington), PROMOTER (Center for the Analysis of Genome Evolution and Function, University of Toronto, Canada), AGRIS (Arabidopsis Gene Regulatory Information Server, Ohio State University, Ohio). Putative abscisic acid response elements (ABRE) and GA-responsive elements (GARE) were identified depending on the threshold e-value and the consensus sequence.

Analysis of ABRE in *TIR1* promoter

The putative ABRE sequence is located 141 bp up-stream of the putative transcription starting point of the *TIR1* gene (Bülow et al., 2010). In order to generate *TIR1_{mABRE}::GUS* recombinant construct containing mutated ABRE sequence, a 2 kb fragment from the promoter region of *TIR1* was amplified using *TIR1*p SalI F 5' CACCGTCGACGAGTA CGAAACCCGAGACTAGG 3' and *TIR1*p EcoRI R 5'

AAAGAATTCCCTCGAGATC TCGATGATCG 3' primers. Amplified product was cloned into pENTR vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The "G" nucleotide in the 5th position of the putative ABRE sequence was changed into "C" using site directed mutagenesis kit (Clontech, Mountain View, CA) following the manufacturer's instructions. The following primers were used for the mutagenesis, TIR1 m F 5' GCTTATAAGACACGTCTCATCATCAGAATCG 3' and TIR1 m R 5' CGATTCTGATGATGAGACGTGTCTTATAAGC 3'. Accuracy of wild type and mutated sequences were verified by sequencing, and sequences were cloned into destination vector pHGWF-s7 using LR clonase reaction kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. These constructs were transformed into wild type *Arabidopsis* plants by using *Agrobacterium*-mediated transformation (Oono et al., 1998; Augustus et al., 2003). The T1 seeds were screened on hygromycin-containing medium, and hygromycin resistant seedlings were transferred to soil. T3 seeds that are homozygous for the transgene were selected for further experiments.

Analysis of GARE in *TIR1* promoter

Two putative GAREs are present 449 bp (GARE1) and 734 bp (GARE2) upstream of transcription start site of *TIR1* gene (Bülow et al., 2010). Putative GARE1 and GARE2 in wild type promoter were mutated as described above using following primers, TIR1 P GARE1 F 5' GCTT CTTTTTTTATTGTTTTTTTACCGTCAGATC 3', TIR1 P GARE1 R 5' GATCTGACGGTAAAAAACAATAAAAAAAGAAGC 3', TIR1 P GARE2 F 5' CGAAAACACTGATTCTTTTTATGTTAATTCATC 3', TIR1 P GARE2 R 5' GATGAATTAACATAAAAAAGAATCAGTGTTTTTCG 3'. Constructs were then

cloned into pHGWF-s7 destination vector as described above. All the constructs were sequenced to confirm the presence of mutations. Wild type (Col-0) plants were transformed with these constructs as described above.

Image Acquisition

For confocal microscopy, images of roots carrying *miR393a::GFP* and *miR393b::GFP* were acquired using an Olympus FV1000 confocal microscope and analyzed using Olympus Fluoview software (Olympus, Melville, NY). Gain and dynamic range settings were calibrated on control GFP expressing roots and then kept unchanged for recording of images of the roots with various treatments. For light microscopy, images of GUS stained seedlings were photographed using Nikon SMZ1500 stereo microscope (Nikon, Melville, NY).

Data analysis

For statistical comparison of quantitative β -glucuronidase assay data, single factor ANOVA was done using “R” software (version 2.13.2, The R Foundation for Statistical Computing, ISBN 3-900051-07-0)

CHAPTER III

RESULTS

TIR1 expression is regulated by salinity stress

To study the effect of salinity stress on the expression of *TIR1* gene, transgenic *Arabidopsis* lines carrying either transcriptional (*TIR1::GUS*) or translational (*TIR1::TIR1-GUS*) gene constructs were treated with various concentrations of NaCl ranging from 0 to 300 mM. Salinity induces *GUS* reporter gene expression (Figure 2.1 a,b,c), suggesting that *TIR1* transcription is induced by salinity. However *TIR1::GUS* expression in roots and shoots shows some differences. At very high NaCl concentration (300 mM) the expression of *TIR1::GUS* is down-regulated in roots, but the same concentration up-regulates its expression in shoots. The expression of *TIR1::TIR1-GUS* was generally less compared to the *TIR1::GUS* expression. *TIR1::TIR1-GUS* is also induced by NaCl up to 200 mM concentration and down-regulated thereafter (Figure 2.2 a,b). Unlike the transcriptional construct, *TIR1::TIR1-GUS* is down-regulated in the shoot at the highest NaCl concentration tested (300 mM).

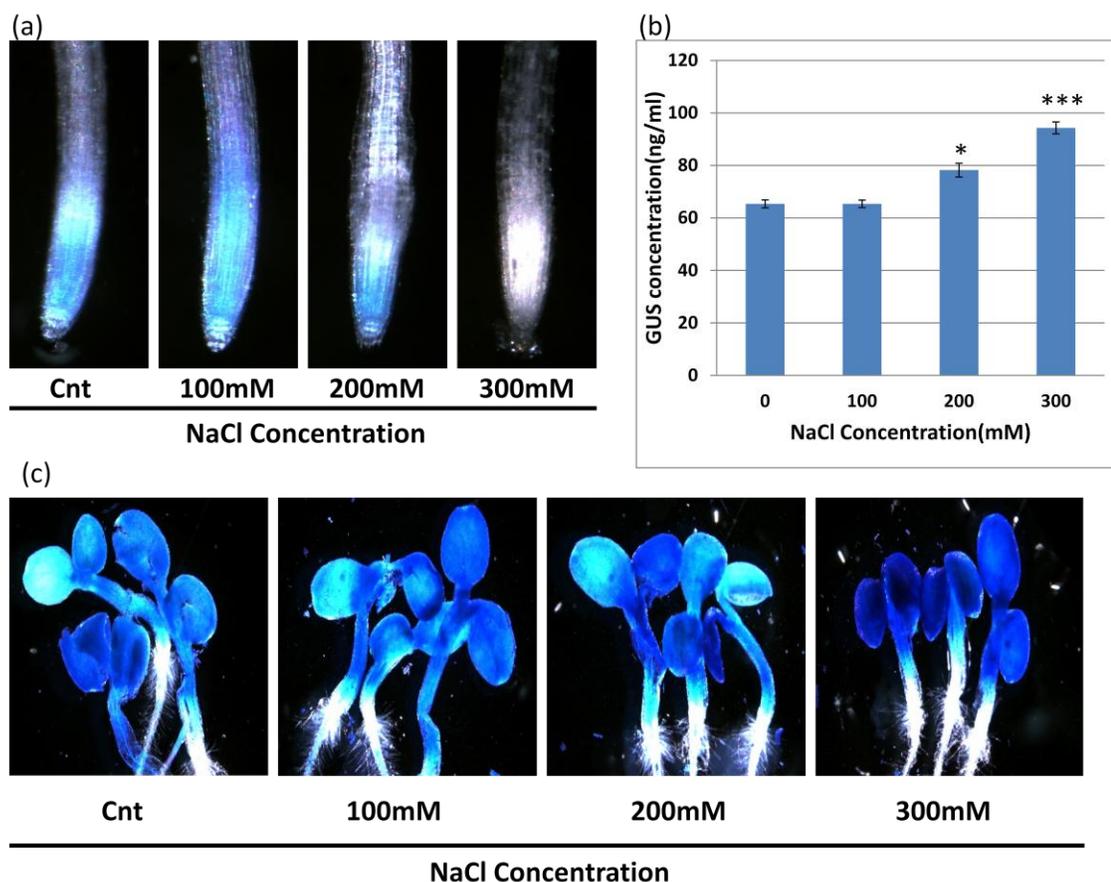


Figure 2.1. *TIR1::GUS* expression is modulated in response to salinity stress. (a) Roots of four day old *TIR1::GUS* transgenic seedlings were treated with various concentrations of NaCl. Seedlings were fixed after the NaCl treatment and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used for measuring GUS activity using MUG assay. Each point indicates the mean value of 3 replicates. Error bars indicate standard deviation of the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”). (c) *TIR1::GUS* expression of shoots of four day old seedlings treated with indicated concentrations of NaCl.

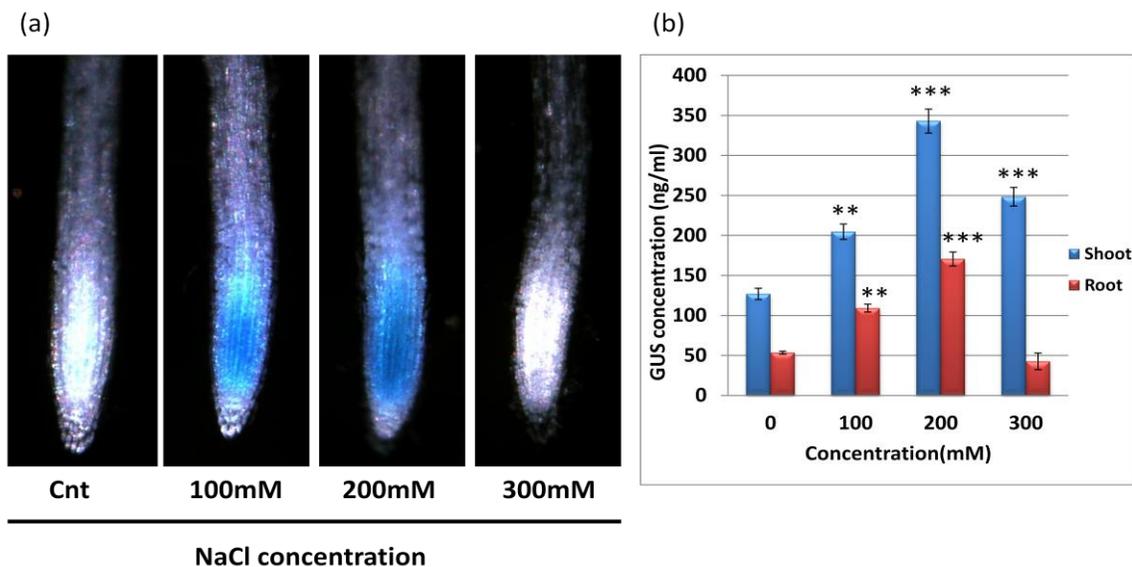


Figure 2.2. *TIR1::TIR1-GUS* expression in response to salinity stress. (a) The expression of *TIR1::TIR1-GUS* in four day old transgenic seedlings treated with NaCl and stained for GUS activity. (b) Quantitative analysis of *TIR1::TIR1-GUS* expression of shoots and roots in response to salinity stress. Roots and shoots of four day old transgenic seedlings carrying *TIR1::TIR1-GUS* were collected separately after NaCl treatments and the quantitative GUS assay was performed as described in methods. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the respective control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

TIR1 expression is regulated by osmotic stress

As NaCl causes both salinity and osmotic stresses, similar experiments were carried out using mannitol which mainly causes osmotic stress. With respect to *TIR1::GUS*, mannitol did not have a significant effect on its expression (Figure 2.3.a,b). However, *TIR1::TIR1-GUS* expression was induced (Figure 2.4. a,b) with increasing

concentrations of mannitol. Nevertheless, compared to mannitol, NaCl induced *TIR1::TIR1-GUS* expression to a higher level suggesting that the induction by NaCl is mainly due to salinity stress.

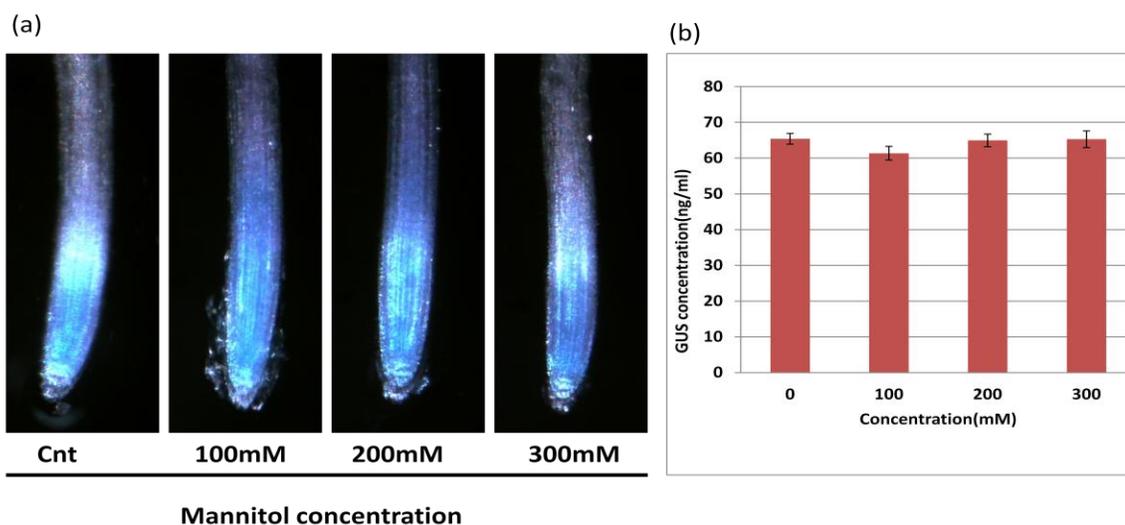


Figure 2.3. *TIR1::GUS* expression in response to osmotic stress. (a) Four day old transgenic seedlings were treated with various concentrations of mannitol and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedling extracts were used in quantitative analysis using MUG assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. There is no significant difference between control and the treatments.

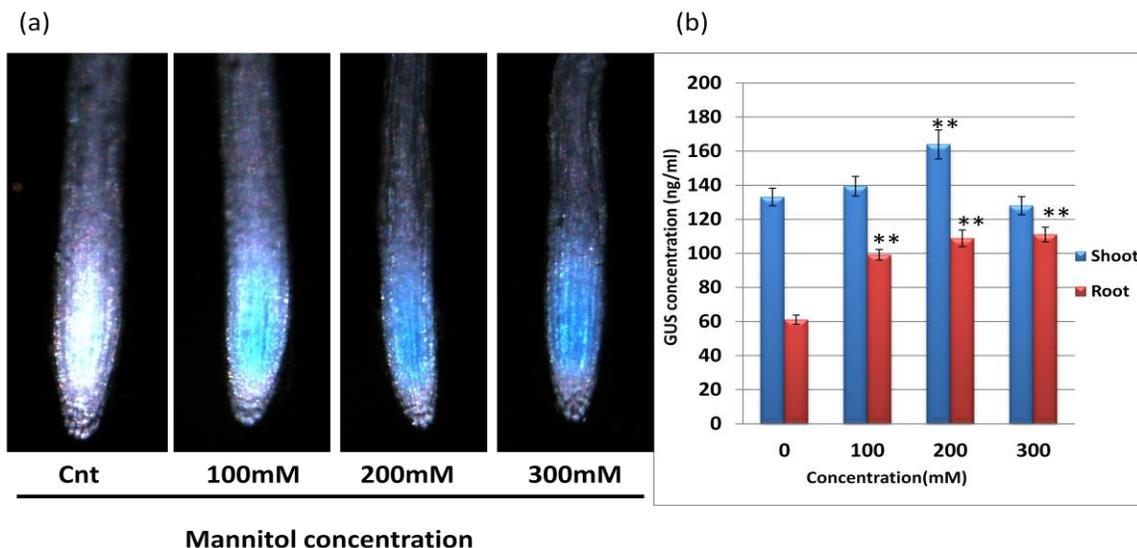


Figure 2.4. *TIR1::TIR1-GUS* expression in response to mannitol. (a) Four day old transgenic seedlings were treated with various concentrations of mannitol and stained for GUS activity. (b) Quantitative analysis of *TIR1::TIR1-GUS* expression of shoots and roots in response to mannitol. Roots and shoots of four day old transgenic seedlings carrying *TIR1::TIR1-GUS* were collected separately after mannitol treatments and quantitative GUS assay was performed. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the respective control ($p < 0.05$ – “**”; $p < 0.01$ – “***”; $p < 0.001$ – “****”).

ABA modulates the expression of *TIR1*

ABA is induced by salinity and osmotic stresses (Koornneef et al., 1998).

Therefore, to study the effects of ABA on *TIR1* expression similar experiments were carried out as described above. *TIR1::GUS* expression is significantly induced by ABA at low concentrations, but it is down-regulated at high concentrations (Figure 2.5.a,b). A

similar induction pattern can be seen in roots expressing *TIR1::TIR1-GUS*. However in shoots, *TIR1::TIR1-GUS* expression is down-regulated by exogenous ABA treatment (Figure 2.6.a,b).

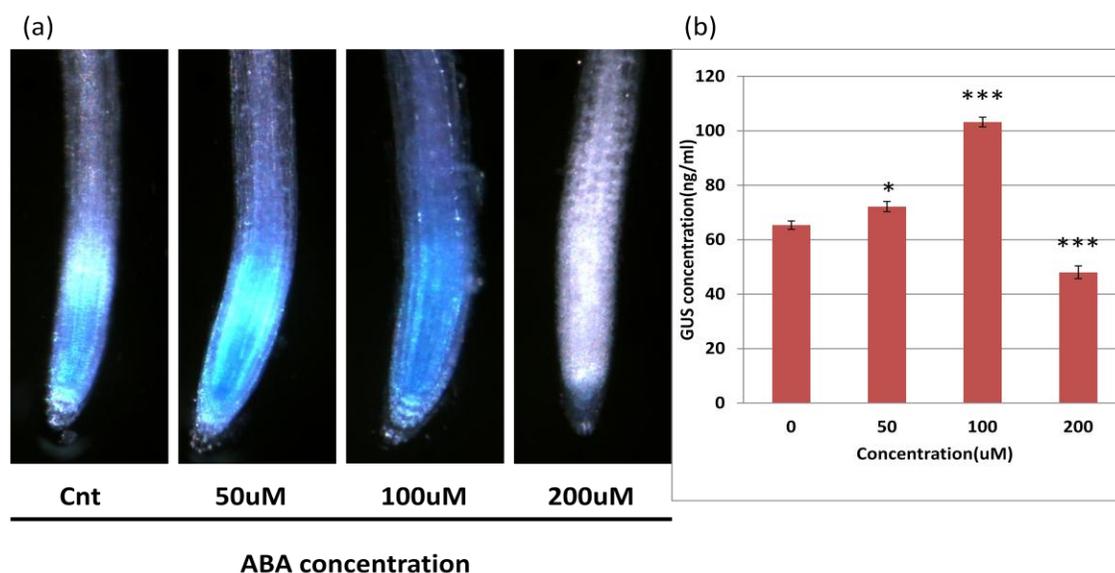


Figure 2.5. Expression of *TIR1::GUS* in response to ABA. (a) Four day old *TIR1::GUS* transgenic seedlings were treated with various concentrations of ABA and then stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

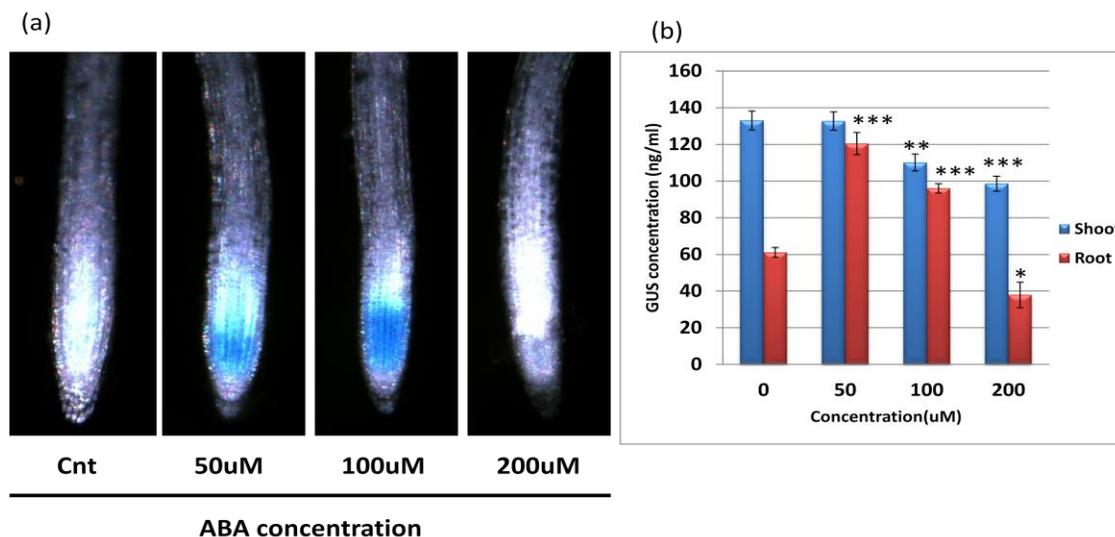


Figure 2.6. Expression of *TIR1::TIR1-GUS* in response to ABA. (a) Four day old *TIR1::TIR1-GUS* transgenic seedlings treated with various concentrations of ABA were stained for GUS activity. (b) Expression of *TIR1::TIR1-GUS* in shoots and roots in response to exogenous ABA treatments. Roots and shoots of four day old transgenic seedlings carrying *TIR1::TIR1-GUS* were collected separately after ABA treatments and used to perform the quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the respective control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

Expression of *TIR1* is regulated by GA

Previous studies show that genes that are regulated by ABA can also be regulated by GA (Busk et al., 1998). As *TIR1* expression is regulated by ABA, similar experiments were carried out to test whether the expression of *TIR1* is controlled by GA. According to both *TIR1::GUS* (Figure 2.7a,b) and *TIR1::TIR1-GUS* expression data, (Figure 2.8a,b)

GA induces GUS expression in these transgenic lines, indicating that expression of *TIR1* is also modulated by GA.

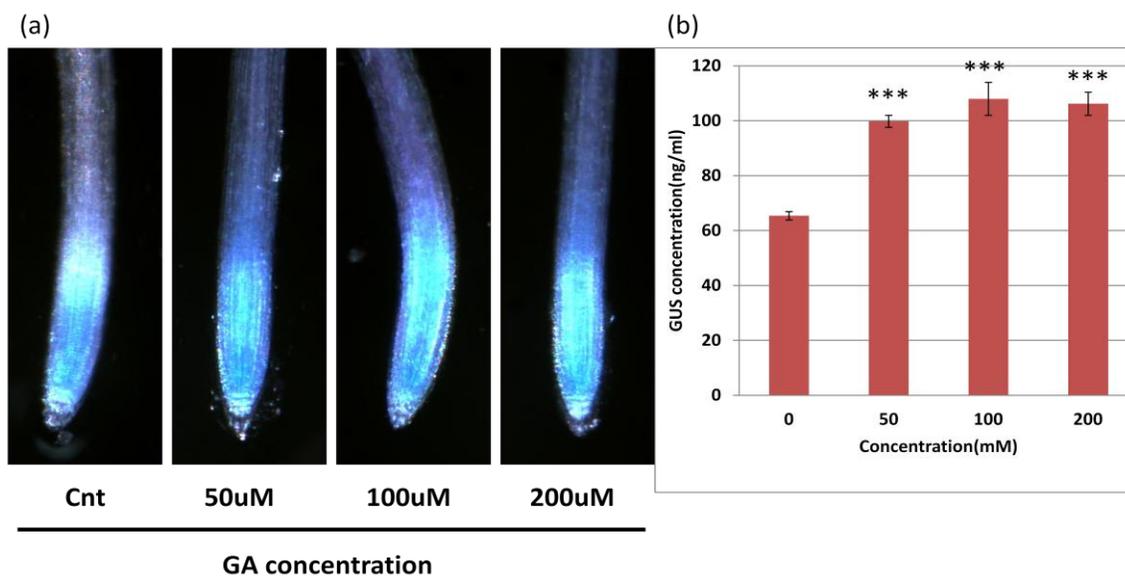


Figure 2.7. Expression of *TIR1::GUS* in response to GA. (a) Four day old *TIR1::GUS* transgenic seedlings were treated with various concentrations of GA and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

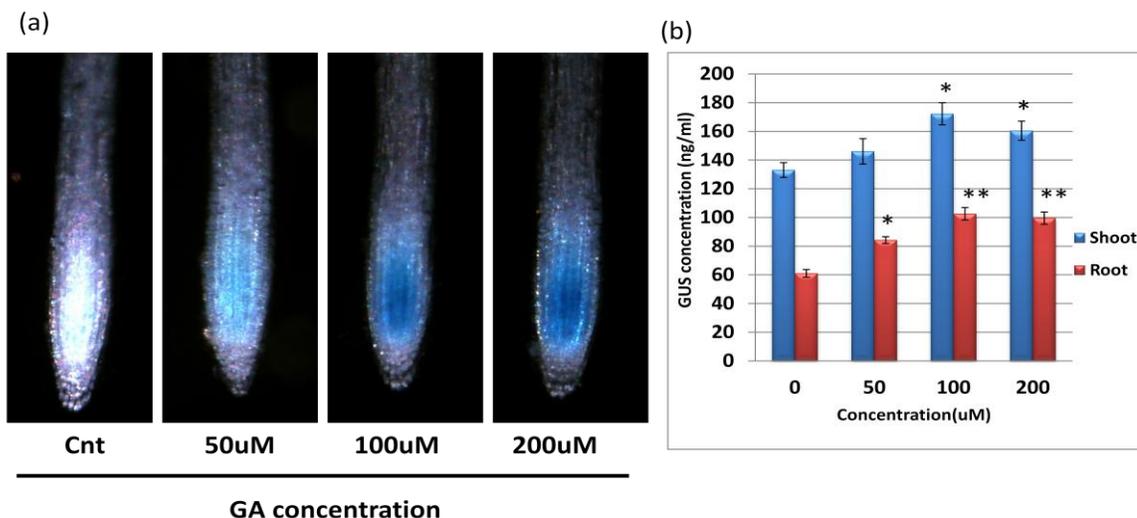


Figure 2.8. Expression of *TIRI::TIRI-GUS* in response to GA. (a) Four day old *TIRI::TIRI-GUS* transgenic seedlings were treated with GA and stained for GUS activity. (b) Expression of *TIRI::TIRI-GUS* in shoots and roots in response to GA. Roots and shoots of four-day old transgenic seedlings carrying *TIRI::TIRI-GUS* were collected separately after GA treatments and quantitative GUS assay was performed. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the respective control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

AFBI expression is modulated by salinity stress

AFBI, a gene closely related to *TIRI*, follows a similar expression pattern as *TIRI* in response to salinity stress, although the maximal expression is seen at a lower NaCl concentration (Figure 3.1.a,b and Figure 3.2.a,b). However, unlike in *TIRI*, *AFBI::AFBI-GUS* expression is down-regulated in roots but up-regulated in shoots at low concentrations of salt (Figure 3.2.c), suggesting the complexity of its regulation.

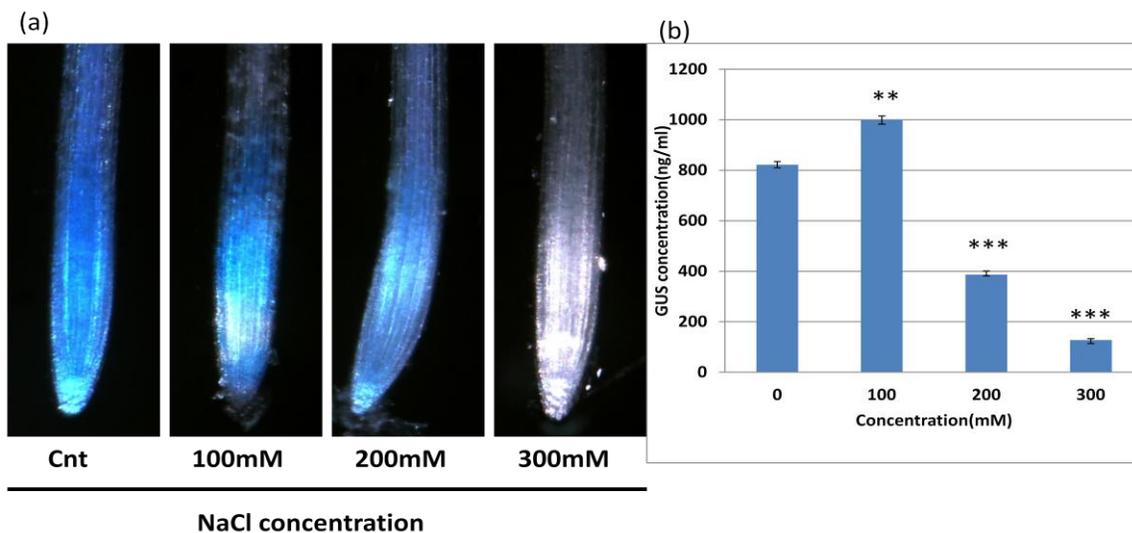


Figure 3.1. Expression of *AFBI::GUS* in response to salinity express. (a) Four day old *AFBI::GUS* transgenic seedlings were treated with NaCl and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

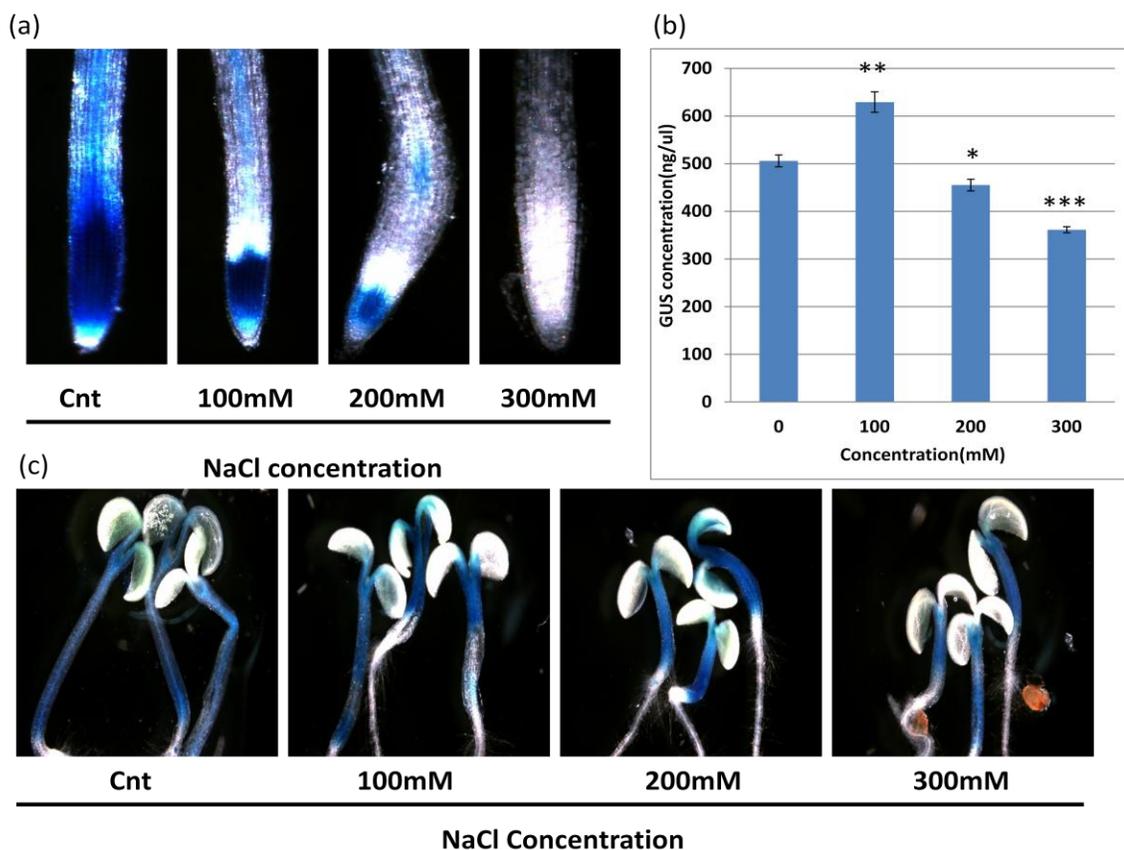


Figure 3.2. Expression of *AFBI::AFBI-GUS* in response to salinity stress. (a) Four day old *AFBI::AFBI-GUS* transgenic seedlings were treated with various concentrations of NaCl and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the respective control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”). (c) Shoots of four day old transgenic seedlings treated with various concentrations of NaCl showing the expression of *AFBI::AFBI-GUS*.

AFBI expression is modulated by osmotic stress

Low concentrations of mannitol up-regulate the expression of *AFBI::GUS* and *AFBI::AFBI-GUS*, but down-regulate expression at higher concentrations (300 mM) (Figure 3.3.a,b and Figure 3.4.a,b). Roots and shoots show a similar expression pattern in both *AFBI::GUS* and *AFBI::AFBI-GUS* in response to osmotic stress.

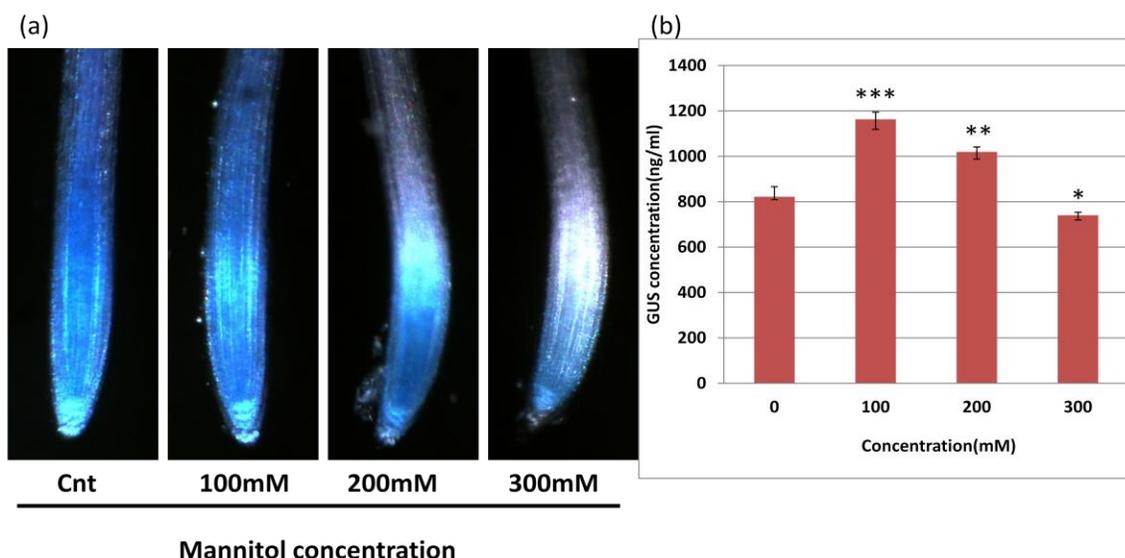


Figure 3.3. *AFBI::GUS* expression in response to osmotic stress. (a) Four day old transgenic seedlings were treated with various concentrations of mannitol and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedling extracts were used in quantitative analysis. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

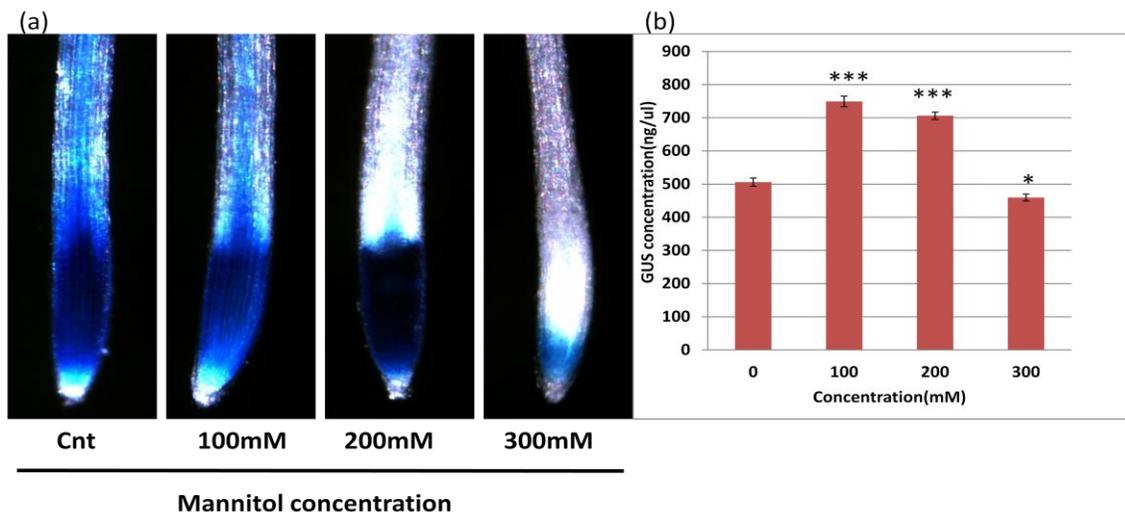


Figure 3.4. *AFBI::AFBI-GUS* expression in response to osmotic stress. (a) Four day old transgenic seedlings were treated with various concentrations of mannitol and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedling extracts were used in quantitative analysis. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

AFBI expression is regulated by ABA

In contrast to the regulation of *AFBI* by NaCl and mannitol, it is down-regulated by ABA in a concentration dependent manner. The reduced expression is evident in both *AFBI::GUS* and *AFBI::AFBI-GUS* reporter constructs (Figure 3.5.a,b and 3.6.a,b).

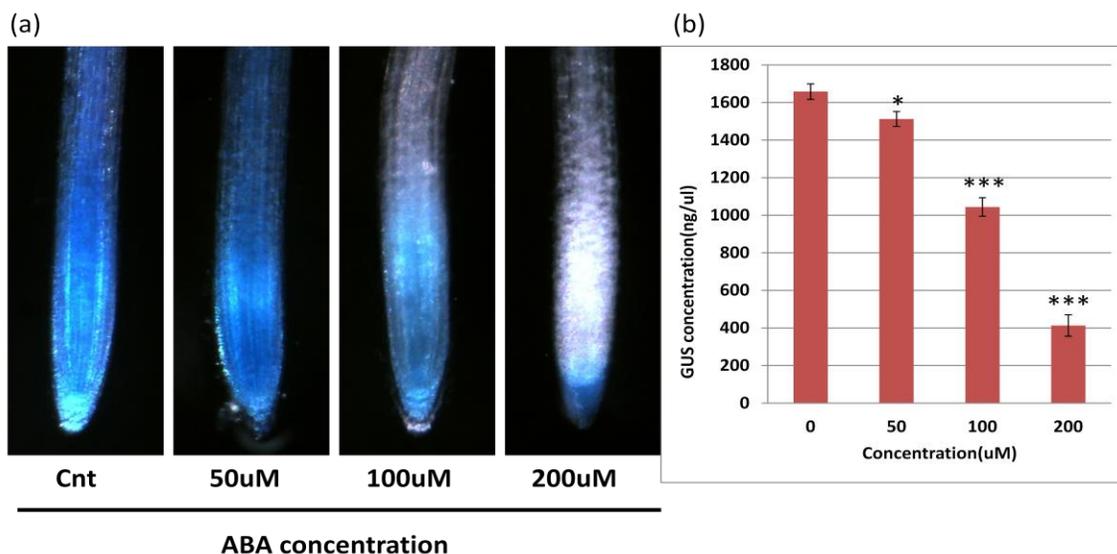


Figure 3.5. Expression of *AFBI::GUS* in response to ABA. (a) Four day old *AFBI::GUS* transgenic seedlings were treated with various concentrations of ABA and then stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

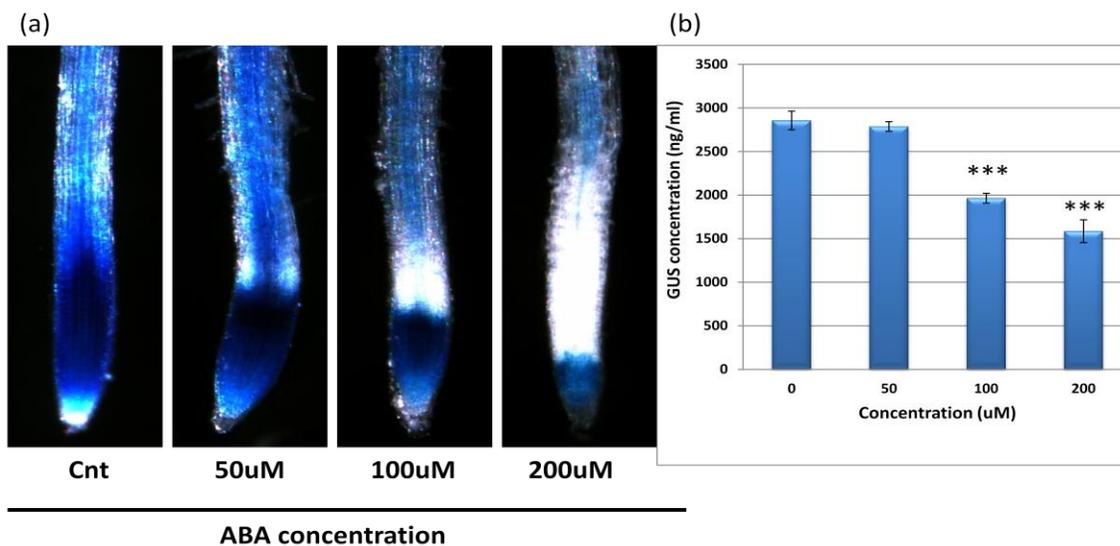


Figure 3.6. Expression of *AFBI::AFB-GUS* in response to ABA. (a) Four day old *AFBI::AFB-GUS* transgenic seedlings were treated with various concentrations of ABA and then stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “**”; $p < 0.01$ – “***”; $p < 0.001$ – “****”).

AFBI expression is regulated by GA

The expression of both *AFBI::GUS* and *AFBI::AFB-GUS* is up-regulated by GA up to 100 μM concentration. At very high concentrations (200 μM), GA down-regulates *GUS* expression suggesting the involvement of GA in the regulation of *AFBI* gene. (Figure 3.7.a,b and Figure 3.8.a,b).

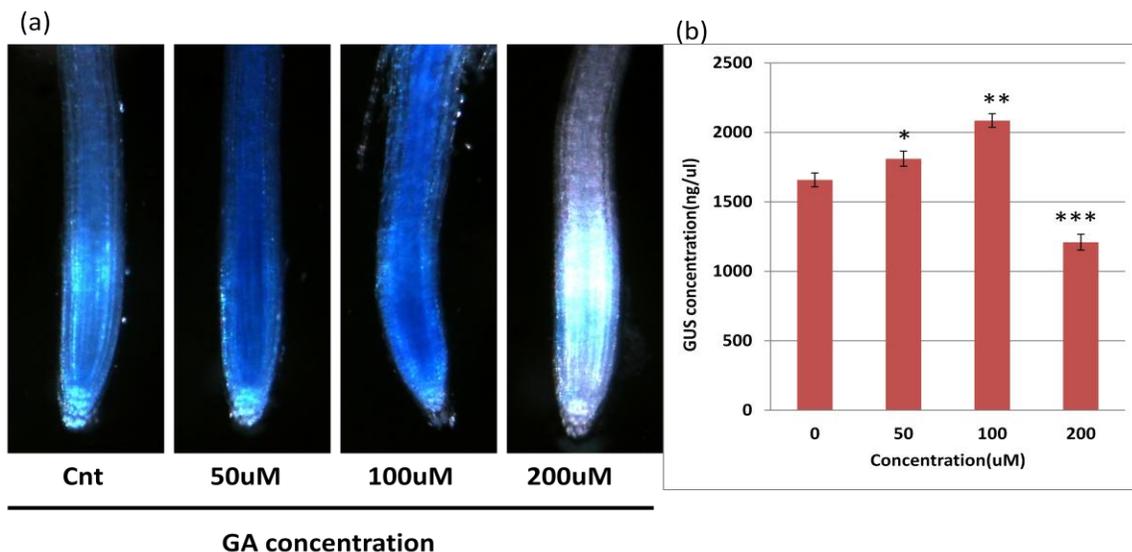


Figure 3.7. Expression of *AFBI::GUS* in response to GA. (a) Four day old *AFBI::GUS* transgenic seedlings were treated with various concentrations of GA and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

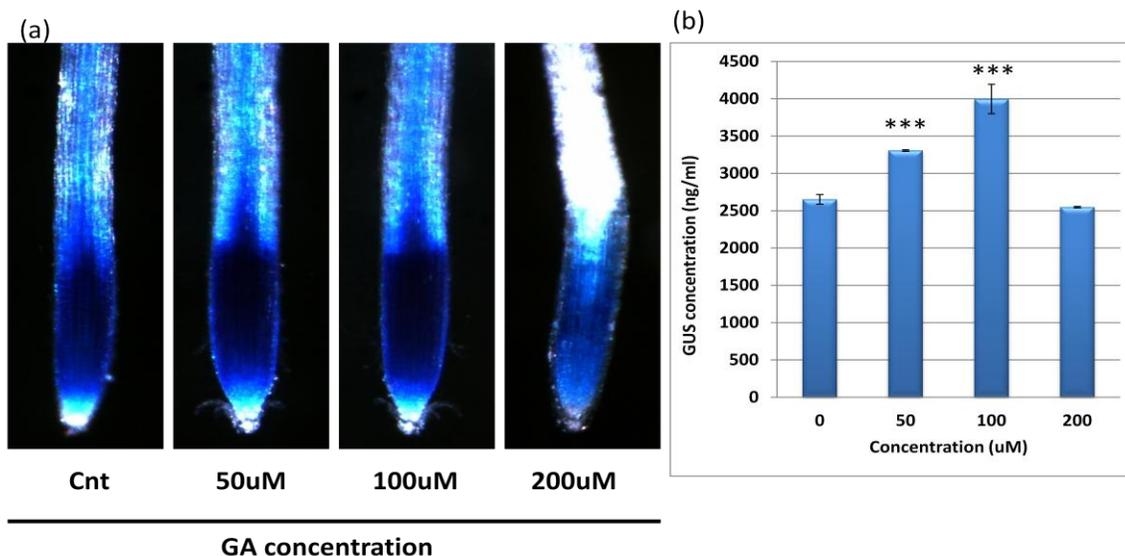


Figure 3.8. Expression of *AFB1::AFB1-GUS* in response to GA. (a) Four day old *AFB1::AFB1-GUS* transgenic seedlings were treated with various concentrations of GA and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “**”; $p < 0.01$ – “***”; $p < 0.001$ – “****”).

Salinity modulates the expression of *AFB2*

Unlike *TIR1* and *AFB1*, *AFB2* is not induced by salinity stress even at low concentrations as shown by reporter gene constructs. *AFB2::GUS* expression (Figure 4.1.a,b) is dramatically down-regulated with increasing NaCl concentration. *AFB2::AFB2-GUS* expression is very low. However, it is also down-regulated by NaCl (Figure 4.2.a,b). The expression patterns in both shoots and roots are similar in all

treatments (shoot data not shown).

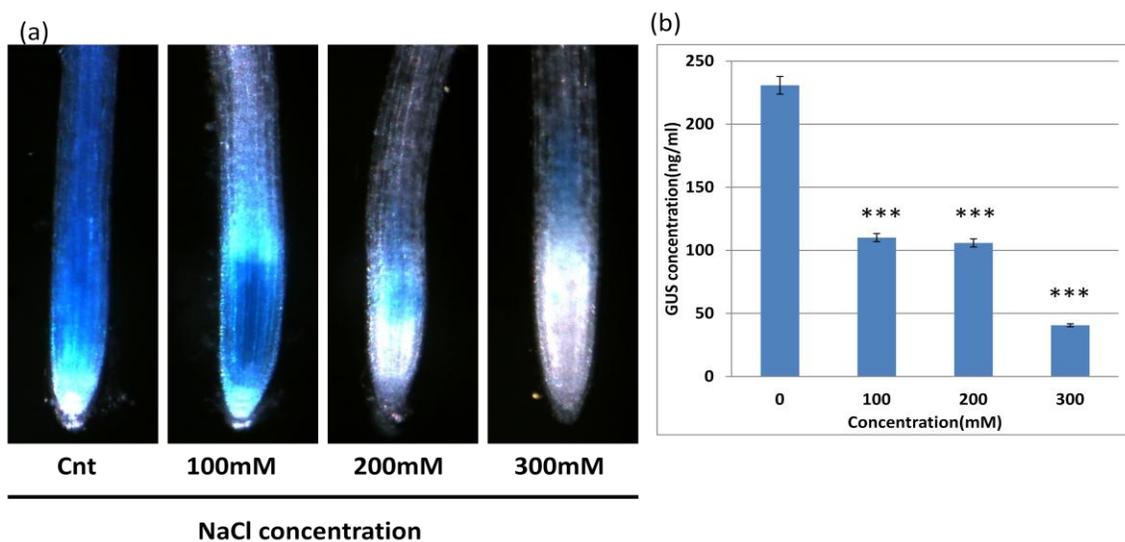


Figure 4.1. *AFB2::GUS* expression in response to salinity stress. (a) Four day old *AFB2::GUS* transgenic seedlings were treated with various concentrations of NaCl. Seedlings were fixed after the NaCl treatment and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

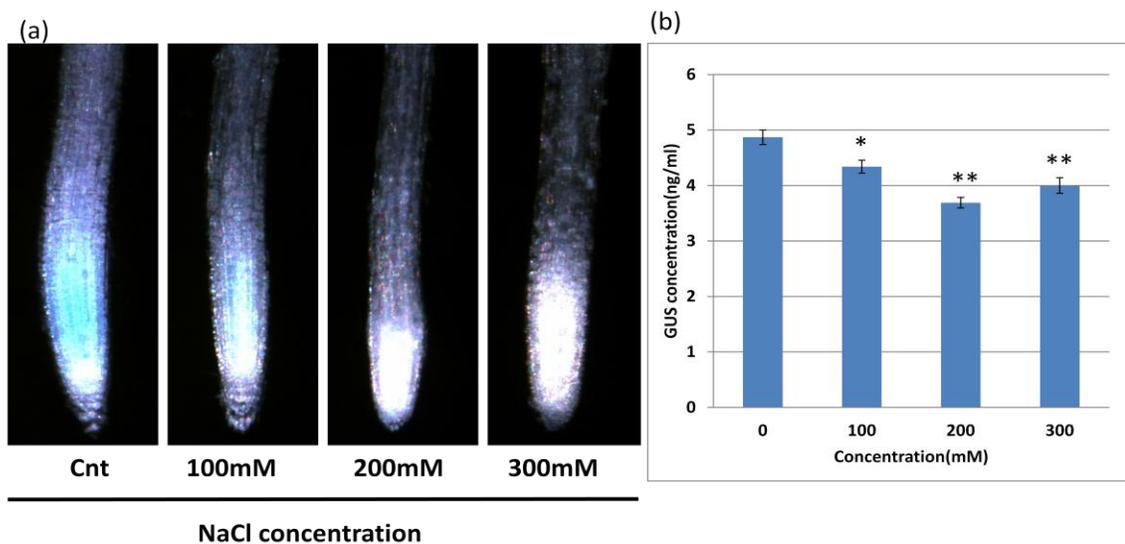


Figure 4.2. *AFB2::AFB2-GUS* expression in response to salinity stress. (a) Four day old *AFB2::AFB2-GUS* transgenic seedlings treated with various concentrations of NaCl. Seedlings were fixed after the NaCl treatment and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

AFB2 expression is regulated by osmotic stress

Similar to salinity stress, osmotic stress also down-regulates *AFB2* expression according to both *AFB2::GUS* (Figure 4.3.a,b) and *AFB2::AFB2-GUS* expression (Figure 4.4.a,b) under different mannitol treatments.

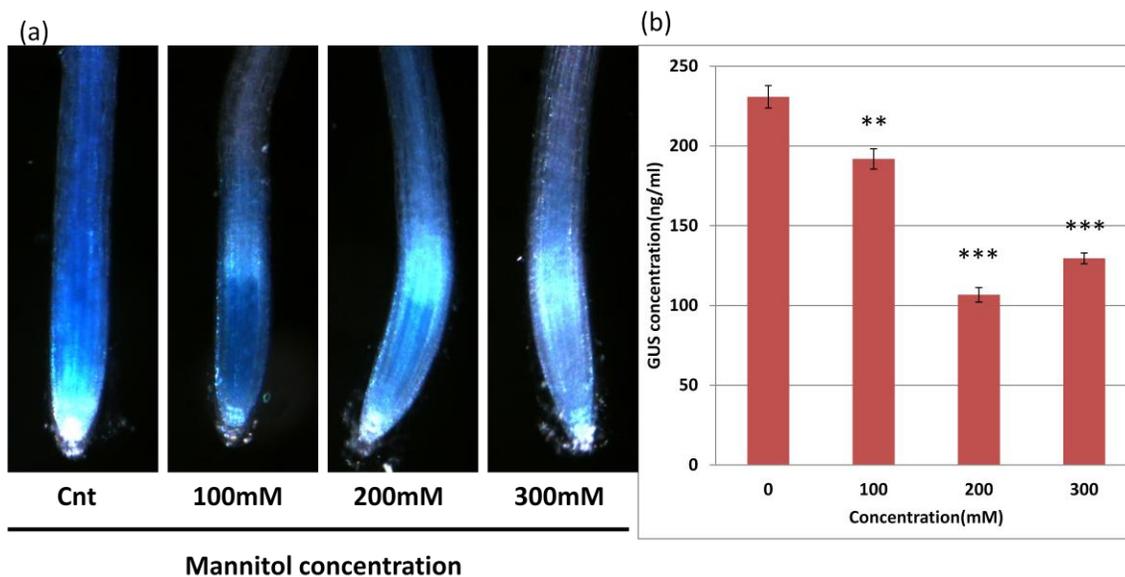


Figure 4.3. *AFB2::GUS* expression in response to osmotic stress. (a) Four day old transgenic seedlings were treated with various concentrations of mannitol and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedling extracts were used in quantitative GUS analysis. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “**”; $p < 0.01$ – “***”; $p < 0.001$ – “****”).

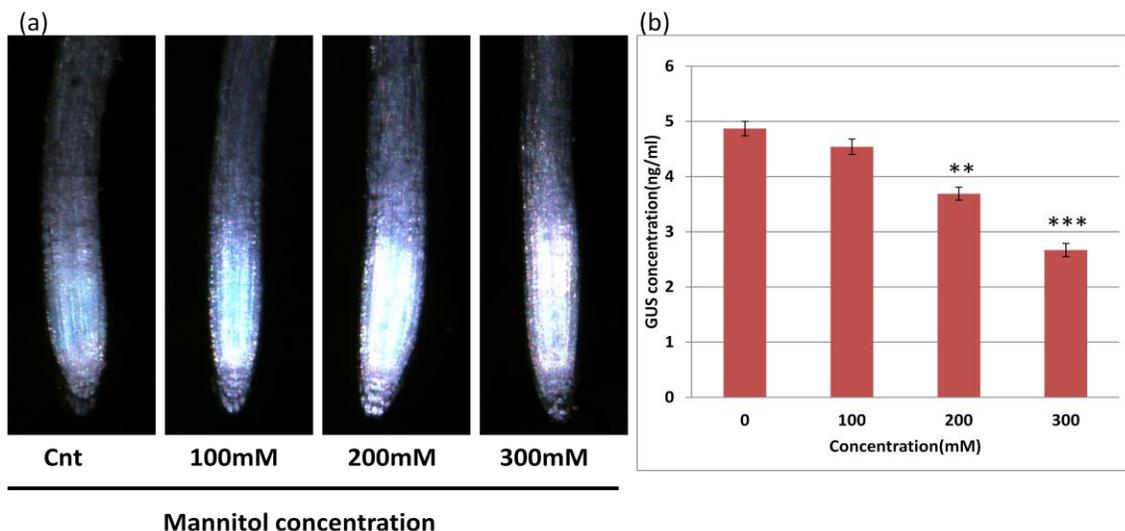


Figure 4.4. *AFB2::AFB2-GUS* expression in response to osmotic stress. (a) Four day old transgenic seedlings were treated with various concentrations of mannitol and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedling extracts were used in quantitative analysis. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

AFB2 expression is regulated by ABA

Similar to salinity and osmotic stress ABA also down-regulates *AFB2* expression in both *AFB2::GUS* and *AFB2::AFB2-GUS* transgenic lines (Figure 4.5.a,b and Figure 4.6.a,b) indicating that *AFB2* expression is generally down-regulated by stress treatments.

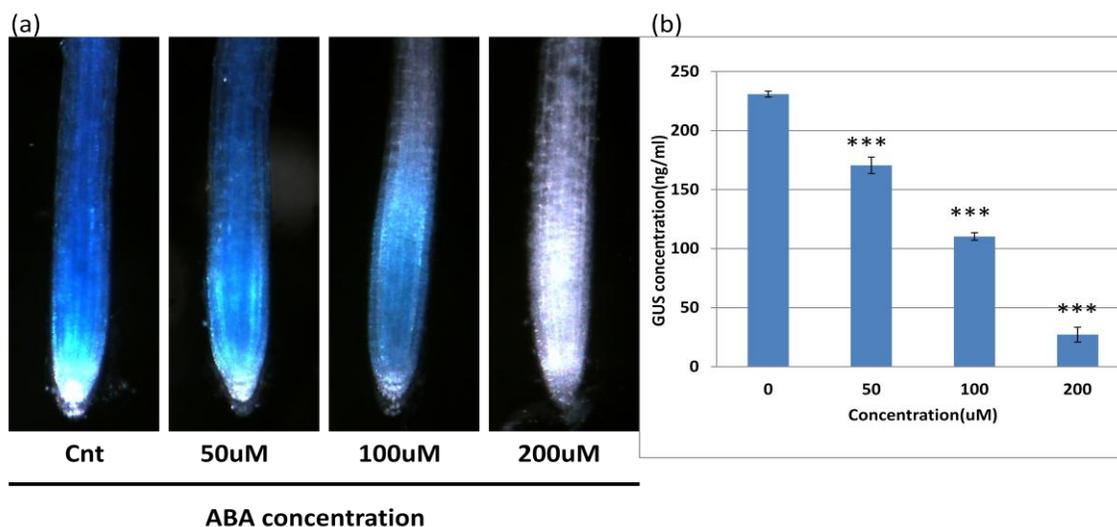


Figure 4.5. Expression of *AFB2::GUS* in response to ABA. (a) Four day old *AFB2::GUS* transgenic seedlings were treated with various concentrations of ABA and then stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for a quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

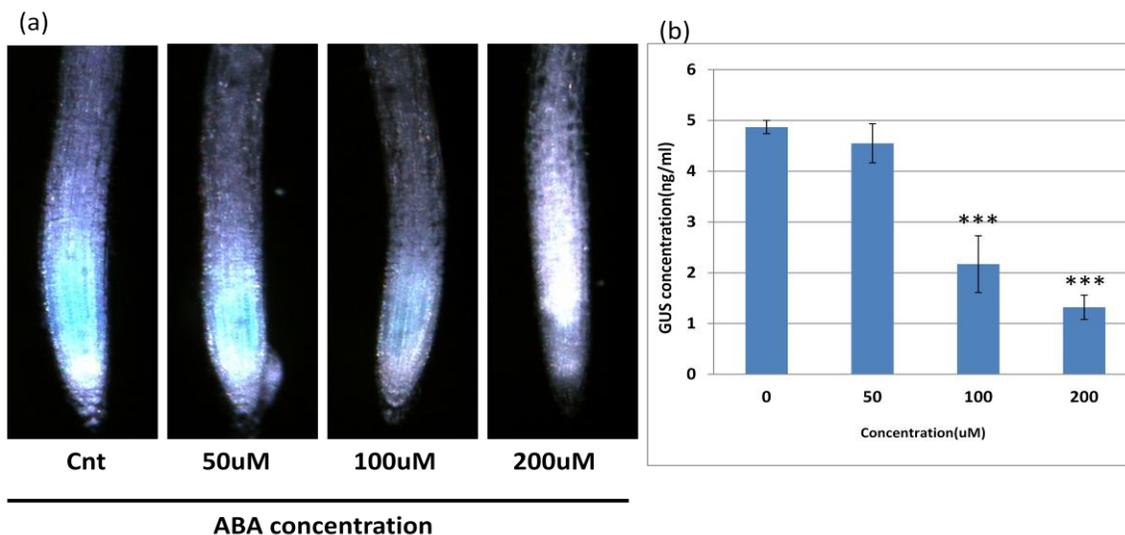


Figure 4.6. Expression of *AFB2::AFB2-GUS* in response to ABA. (a) Four day old *AFB2::AFB2-GUS* transgenic seedlings were treated with various concentrations of ABA and then stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

GA up-regulates the expression of *AFB2*

Compared to other treatments, GA up-regulates the expression of *AFB2::GUS* and *AFB2::AFB2-GUS* as indicated by both histological and quantitative assays (Figure 4.7.a,b and Figure 4.8.a,b). However, at very high concentration of GA, expression of both reporter genes is down-regulated.

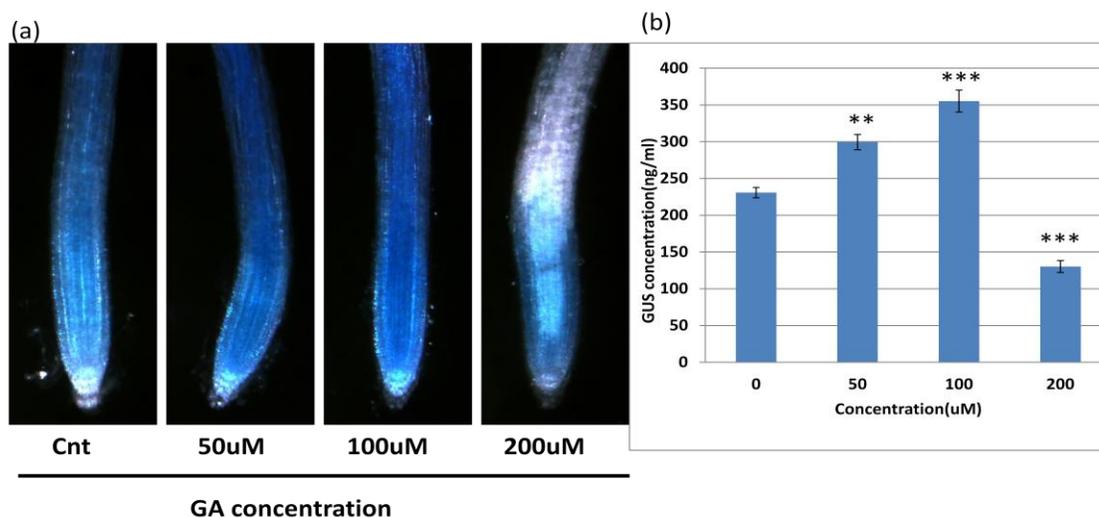


Figure 4.7. Expression of *AFB2::GUS* in response to GA. (a) Four day old *AFB2::GUS* transgenic seedlings were treated with various concentrations of GA and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the respective control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

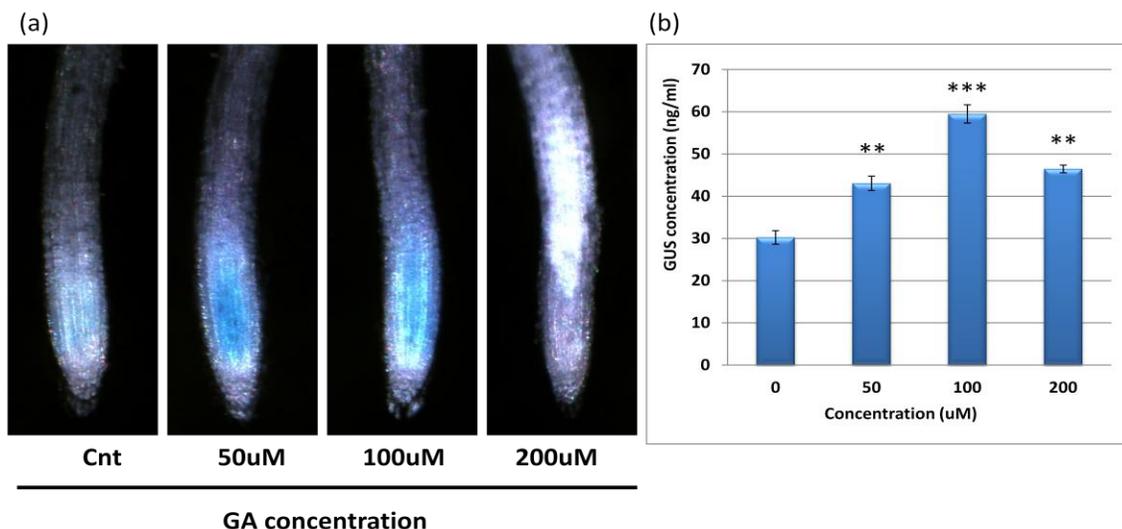


Figure 4.8. Expression of *AFB2::AFB2-GUS* in response to GA. (a) Four day old *AFB2::AFB2-GUS* transgenic seedlings were treated with various concentrations of GA and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

AFB3 expression is modulated by salinity stress

Comparable with expression of *AFB2*, expression of both *AFB3::GUS* and *AFB3::AFB3-GUS* is also significantly down-regulated by salinity stress (Figure 5.1.a,b and Figure 5.2.a,b). A similar trend of down-regulation could be seen in both roots and shoots (shoot data are not shown).

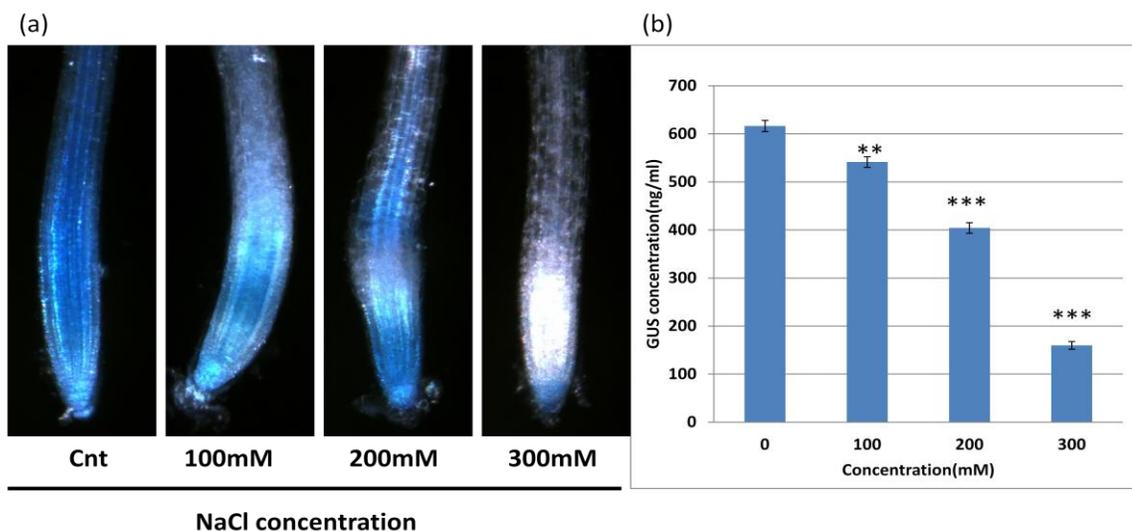


Figure 5.1. *AFB3::GUS* expression in response to salinity stress. (a) Four day old *AFB3::GUS* transgenic seedlings were treated with various concentrations of NaCl. Seedlings were fixed after the NaCl treatment and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

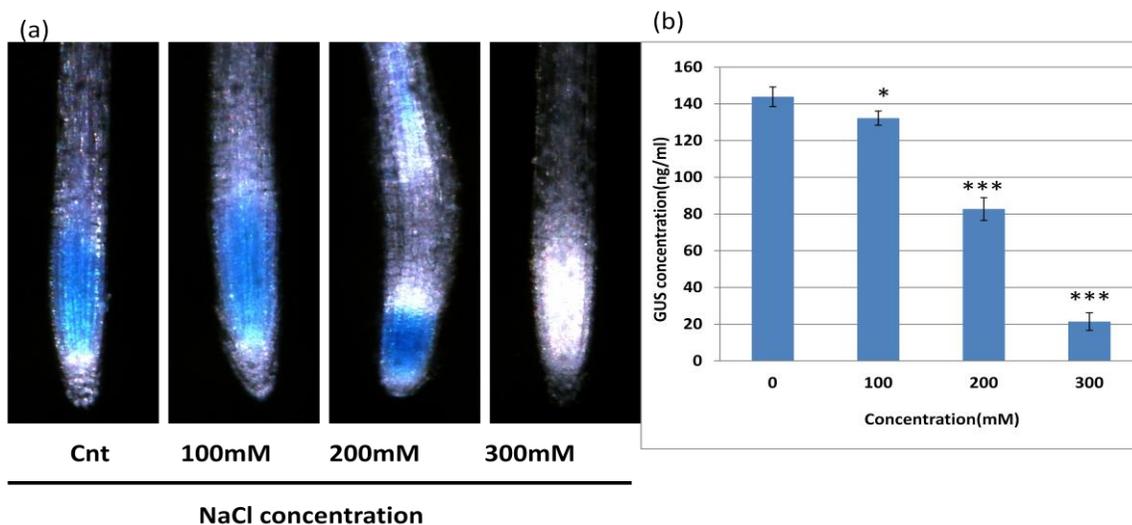


Figure 5.2. *AFB3::AFB3-GUS* expression in response to salinity stress. (a) Four day old *AFB3::AFB3-GUS* transgenic seedlings were treated with various concentrations of NaCl. Seedlings were fixed after the NaCl treatment and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

Osmotic stress regulates the expression of *AFB3*

Osmotic stress down-regulates *AFB3::GUS* expression (Figure 5.3.a,b). However, the expression of *AFB3::AFB3-GUS* is up-regulated at low concentrations of mannitol and then down-regulated at very high concentrations. This dual response again suggests the presence of additional *cis*-regulatory elements necessary for osmotic stress responses within the gene.

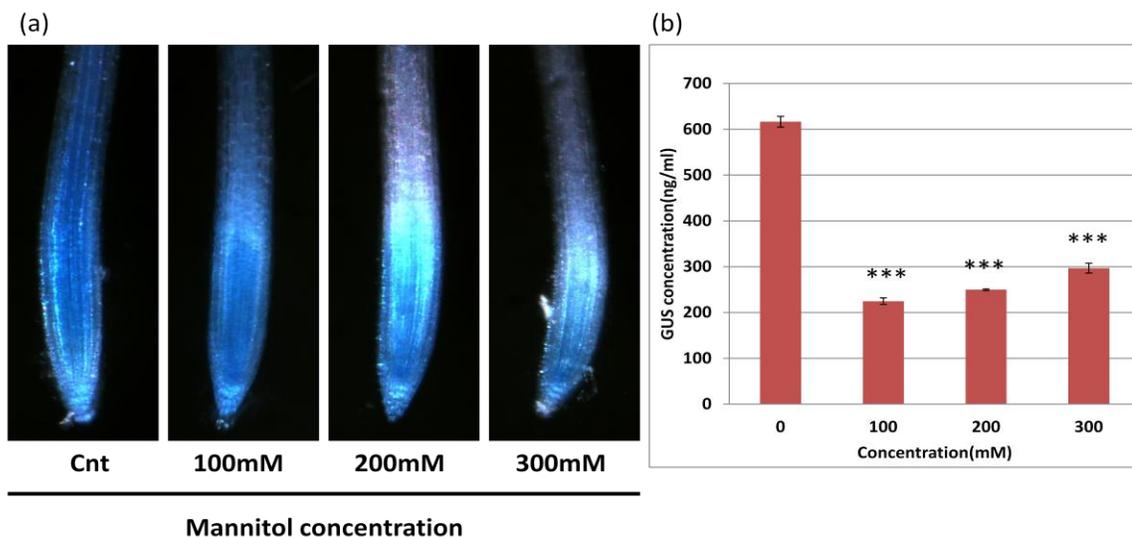


Figure 5.3. *AFB3::GUS* expression in response to osmotic stress. (a) Four day old transgenic seedlings were treated with various concentrations of mannitol and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

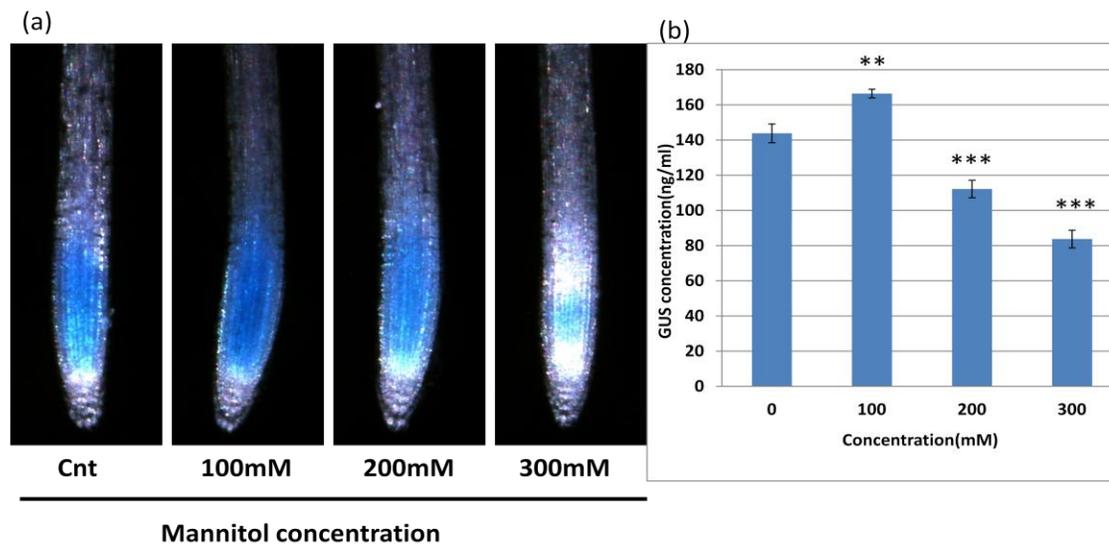


Figure 5.4. *AFB3::AFB3-GUS* expression in response to osmotic stress. (a) Four day old transgenic seedlings were treated with various concentrations of mannitol and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedling extracts were used in quantitative analysis. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

ABA up-regulates the expression of *AFB3*

The expression of *AFB3* fused reporter genes is up-regulated by ABA at low concentrations and down-regulated at high concentrations. This pattern of response is evident in both transcriptional (Figure 5.5.a,b) and translational (Figure 5.6.a,b) reporter constructs.

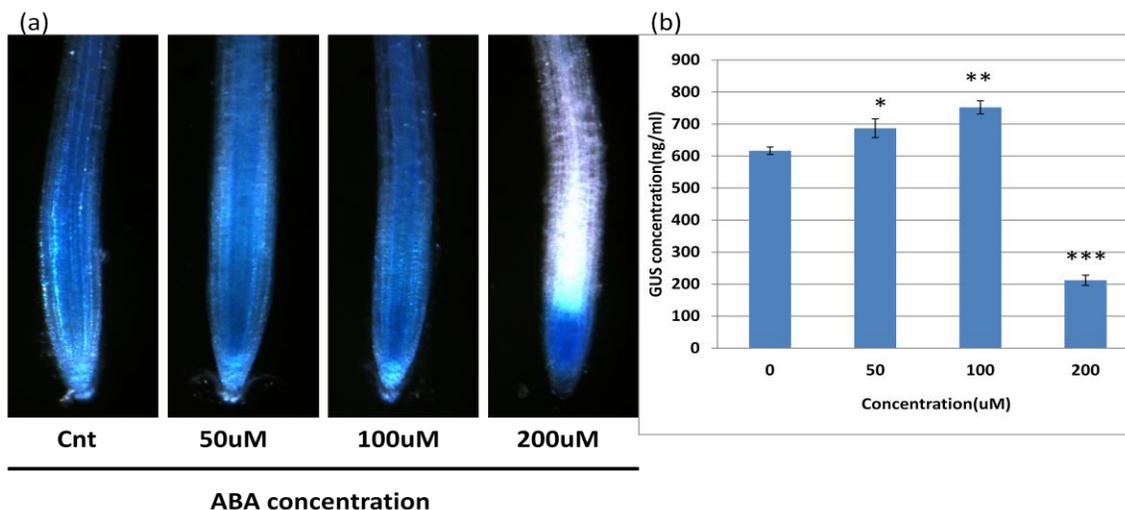


Figure 5.5. Expression of *AFB3::GUS* in response to ABA. (a) Four day old *AFB3::GUS* transgenic seedlings were treated with various concentrations of ABA and then stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

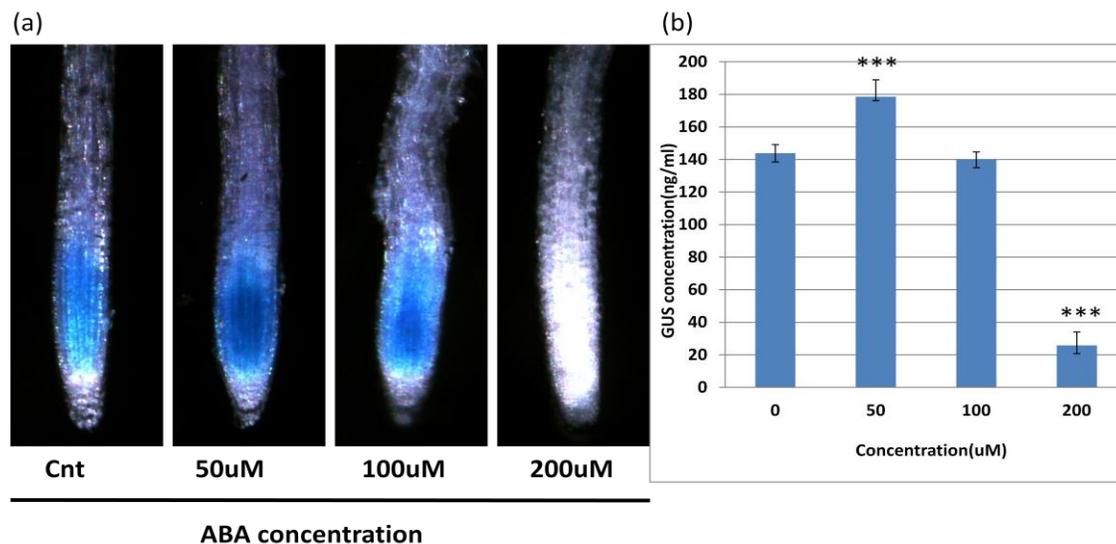


Figure 5.6. Expression of *AFB3::AFB3-GUS* in response to ABA. (a) Four day old *AFB3::AFB3-GUS* transgenic seedlings were treated with various concentrations of ABA and then stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

GA up-regulates the expression of *AFB3*

Following the similar pattern as *TIR1*, *AFB1* and *AFB2* fused reporter genes, the expression of *AFB3::GUS* and *AFB3::AFB3-GUS* is also up-regulated by low concentrations and down-regulated by high concentrations of GA (Figure 5.7.a,b and Figure 5.8.a,b).

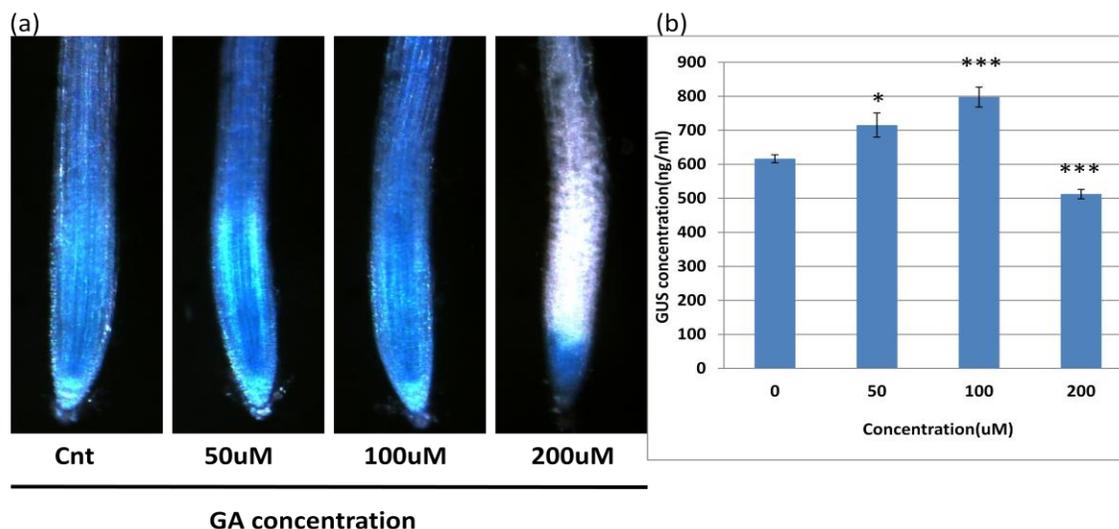


Figure 5.7. Expression of *AFB3::GUS* in response to GA. (a) Four day old *AFB3::GUS* transgenic seedlings were treated with various concentrations of GA and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

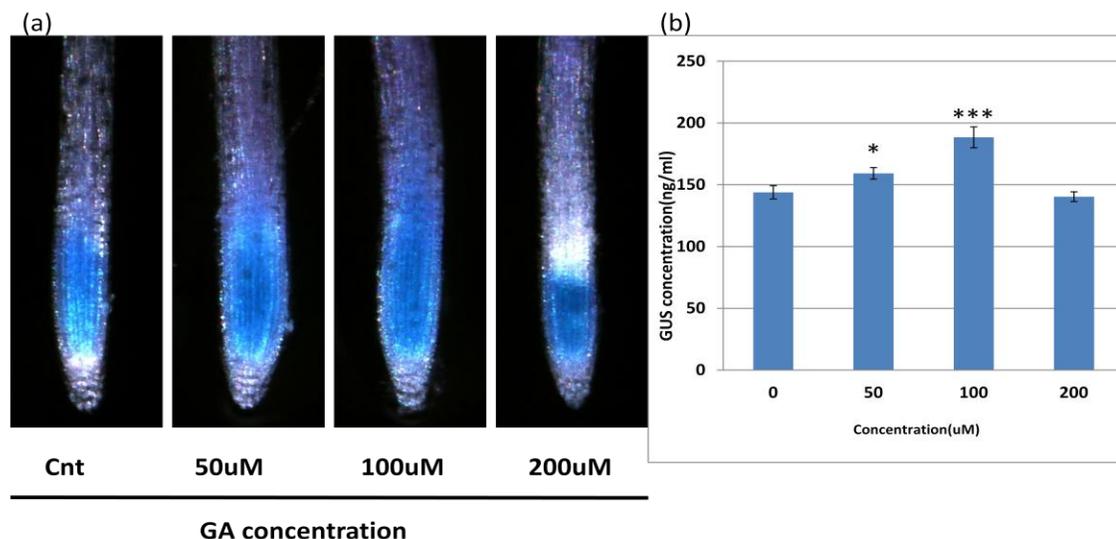


Figure 5.8. Expression of *AFB3::AFB3-GUS* in response to GA. (a) Four day old *AFB3::AFB3-GUS* transgenic seedlings were treated with various concentrations of GA and stained for GUS activity. (b) Quantitative analysis of GUS activity. Whole seedlings were used to extract total protein for quantitative GUS assay. Each data point indicates the mean value of 3 replicates. Error bars indicate standard deviation from the mean. Stars indicate that the means differ significantly from the control ($p < 0.05$ – “*”; $p < 0.01$ – “**”; $p < 0.001$ – “***”).

RT-PCR analysis of auxin receptor genes

RT-PCR results indicate while *TIR1* is slightly up-regulated in response to salinity and osmotic stresses, all other *AFBs* are down-regulated (Figure 6.1 and 6.2). In response to ABA, while *TIR1* and *AFB3* are up-regulated, *AFB1* and *AFB2* are down-regulated (Figure 6.3). GA treatment up-regulates all the receptor genes at low concentrations but down-regulates at high concentrations.

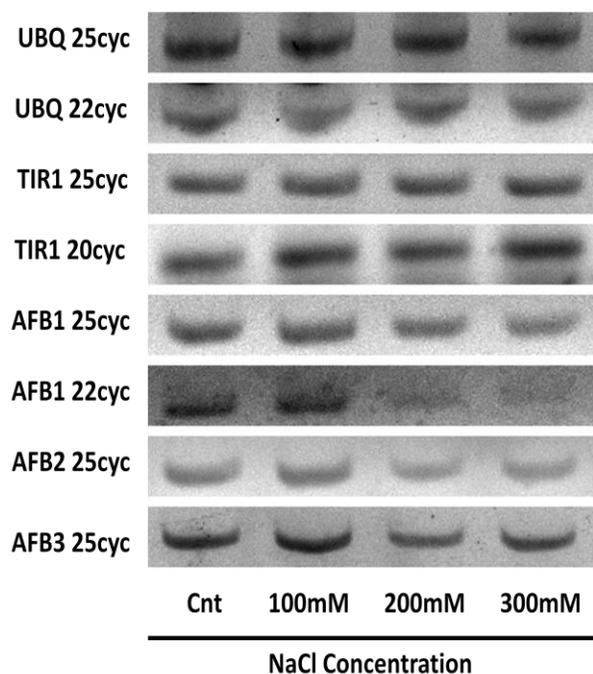


Figure 6.1. RT-PCR analysis of F-box receptor genes in response to salinity stress. Four day old wild type (Col-0) seedlings were treated with NaCl for 18 hrs in liquid ATS medium. Samples were frozen in liquid nitrogen and RNA was isolated. cDNA was synthesized and PCR reactions were carried out using specific internal primers for each auxin receptor gene. Ubiquitin 11 was used as the internal control. Number of amplification cycles used is indicated following the name of the gene.

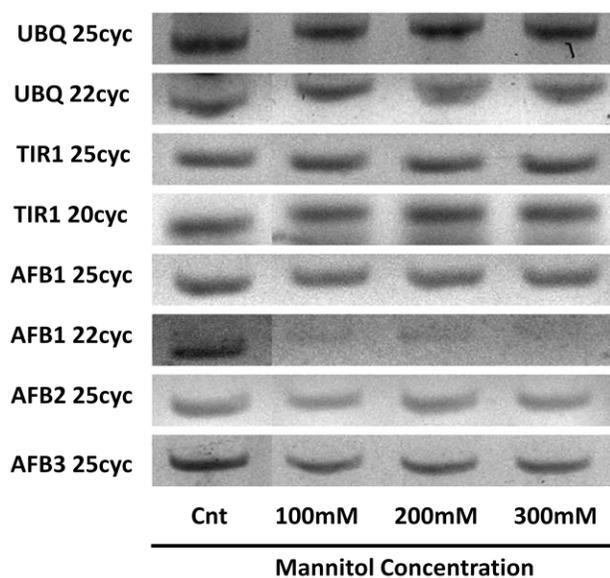


Figure 6.2. RT-PCR analysis of F-box receptor genes in response to osmotic stress. Four day old wild type (Col-0) seedlings were treated with mannitol for 18 hrs in liquid ATS medium. Samples were frozen in liquid nitrogen and RNA was isolated. cDNA was synthesized and PCR reactions were carried out using specific internal primers for each auxin receptor gene. Ubiquitin 11 was used as the internal control. Number of amplification cycles is indicated following the name of the gene.

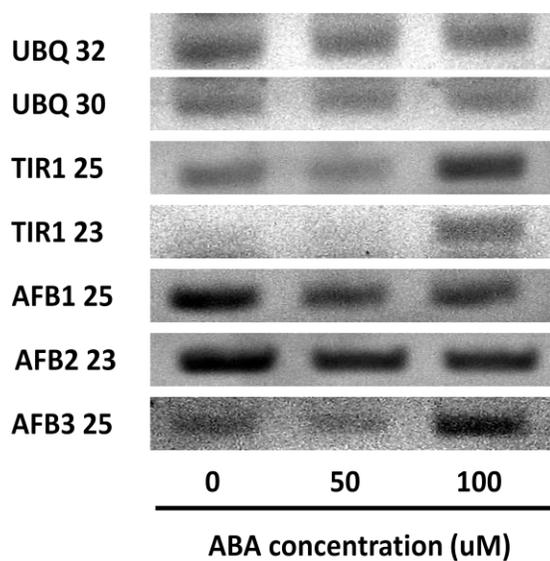


Figure 6.3. RT-PCR analysis of F-box receptor genes in response to ABA. Four day old wild type (Col-0) seedlings were treated with ABA for 18 hrs in liquid ATS medium. Samples were frozen in liquid nitrogen and RNA was isolated. cDNA was synthesized, and PCR reactions were carried out using specific internal primers for each auxin receptor gene. Ubiquitin 11 was used as the internal control. Number of amplification cycles is indicated following the name of the gene.

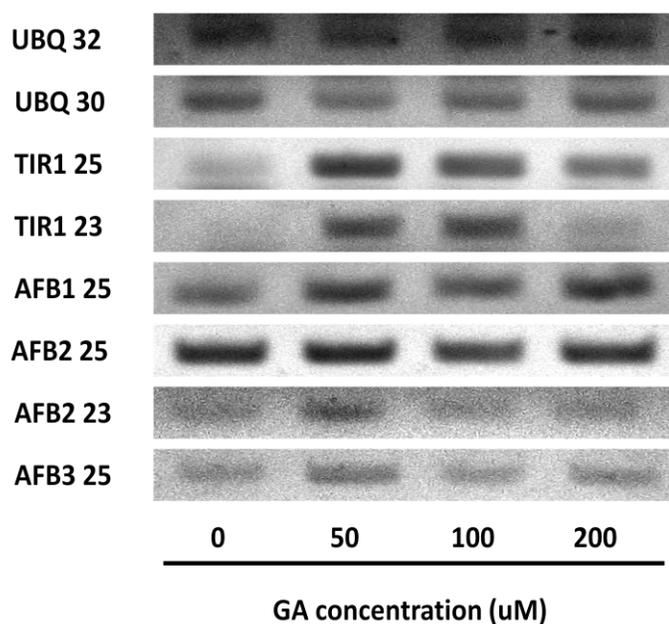


Figure 6.4. RT-PCR analysis of auxin receptor F-box genes in response to GA. Four day old wild type (Col-0) seedlings were treated with GA for 18 hrs in liquid ATS medium. Samples were frozen in liquid nitrogen and RNA was isolated. cDNA was synthesized and PCR reactions were carried out using specific internal primers for each auxin receptor gene. Ubiquitin 11 was used as the internal control. Number of amplification cycles is indicated following the name of the gene.

Experiment	Salinity stress (NaCl)															
	<i>TIR1</i>				<i>AFB1</i>				<i>AFB2</i>				<i>AFB3</i>			
	Concentration(mM)				Concentration(mM)				Concentration(mM)				Concentration(mM)			
	0	100	200	300	0	100	200	300	0	100	200	300	0	100	200	300
Transcriptional reporter gene expression	+	+	++	+++	++	+++	+	+	++	+	+	+	+++	++	+	+
Translational reporter gene expression	+	++	+++	+	++	+++	+	+	++	+	+	+	+++	++	+	+
RT PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 1 continued. “+” indicates the relative expression level. Expression levels among different genes are not comparable.

Table 2. Summary of the expression patterns of auxin receptor genes in response to osmotic stress																
Experiment	Osmotic stress (Mannitol)															
	<i>TIR1</i>				<i>AFB1</i>				<i>AFB2</i>				<i>AFB3</i>			
	Concentration(mM)				Concentration(mM)				Concentration(mM)				Concentration(mM)			
	0	100	200	300	0	100	200	300	0	100	200	300	0	100	200	300
Transcriptional reporter gene expression	++	++	++	++	+	++	++	+	++	++	+	+	++	+	+	+
Translational reporter gene expression	+	++	++	++	+	++	++	+	++	++	+	+	++	++	++	+
RT PCR	+	+	++	++	++	+	+	+	++	+	+	+	++	+	+	+

“+” indicates the relative expression level. Expression levels among different genes are not comparable.

Table 3. Summary of the expression patterns of auxin receptor genes in response to ABA																
Experiment	ABA															
	<i>TIR1</i>				<i>AFB1</i>				<i>AFB2</i>				<i>AFB3</i>			
	Concentration(μM)				Concentration(μM)				Concentration(μM)				Concentration(μM)			
	0	50	100	200	0	50	100	200	0	50	100	200	0	50	100	200
Transcriptional reporter gene expression	+	++	++	+	++	++	+	+	++	++	+	+	+	++	++	+
Translational reporter gene expression	+	++	++	+	++	++	+	+	++	++	+	+	++	++	++	+
RT PCR	+	+	++	++	++	+	+	+	++	+	+	+	+	+	++	++

Table 3 continued. “+” indicates the relative expression level. Expression levels among different genes are not comparable.

Table 4. Summary of the expression patterns of auxin receptor genes in response to GA																
Experiment	GA															
	<i>TIR1</i>				<i>AFB1</i>				<i>AFB2</i>				<i>AFB3</i>			
	Concentration(μM)				Concentration(μM)				Concentration(μM)				Concentration(μM)			
	0	50	100	200	0	50	100	200	0	50	100	200	0	50	100	200
Transcriptional reporter gene expression	+	++	+++	+++	+	++	+++	+	+	++	+++	+	+	++	+++	+
Translational reporter gene expression	+	++	+++	++	+	++	+++	+	+	++	+++	++	+	++	+++	+
RT PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

“+” indicates the relative expression level. Expression levels among different genes are not comparable.

Analysis of *miR393* expression

The *miR393* is a negative regulator of auxin receptor F-box genes. Therefore, the expression of *miR393a* and *miR393b* were examined in response to salinity, osmotic stress, ABA and GA using *miR393a::GFP* and *miR393b::GFP* transgenic seedlings that have been described previously (Navarro et al., 2006).

Expression of *miR393a* is regulated by salinity, osmotic stress, ABA and GA

The expression of *miR393a::GFP* is mainly confined to the peripheral cells of the root. The expression level of *miR393a::GFP* is very low, but clearly the expression can be detected in transgenic seedlings compared to wild type Col-0 (data not shown). The expression of *miR393a::GFP* is considerably induced by salinity stress (Figure 7.1). However, unlike salinity, osmotic stress down-regulates the expression of *miR393a::GFP* (Figure 7.2). While ABA up-regulates *miR393a::GFP* expression at low concentrations, it down-regulates the expression at higher concentrations (Figure 7.3). *miR393a::GFP* follows the same expression pattern in response to GA, except it reaches its maximum at a higher concentration of GA than that of ABA (Figure 7.4). These results suggest that the expression of *miR393a* is regulated by salinity, osmotic stress, ABA and GA.

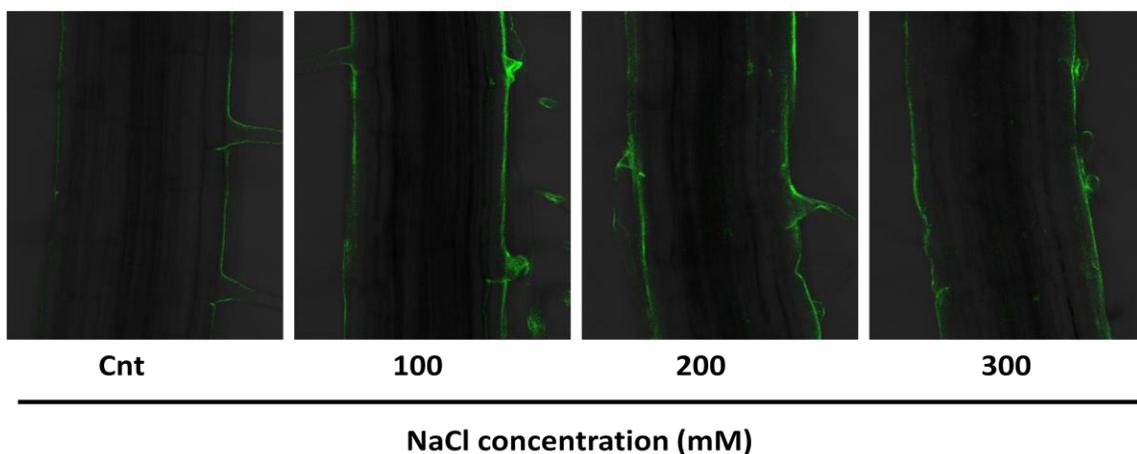


Figure 7.1. Expression of *miR393a::GFP* in response to salinity stress. Four day old transgenic seedlings carrying *miR393a::GFP* gene construct were treated with NaCl for 18 hrs. Expression of *miR393a::GFP* in roots was detected using Olympus FV1000 confocal microscopy. Images represent a single 0.5 μm thick optical section taken in the

middle plane of the root using 20x water immersion lens with a numerical aperture of 1.2, enabling the vascular tissues were imaged.

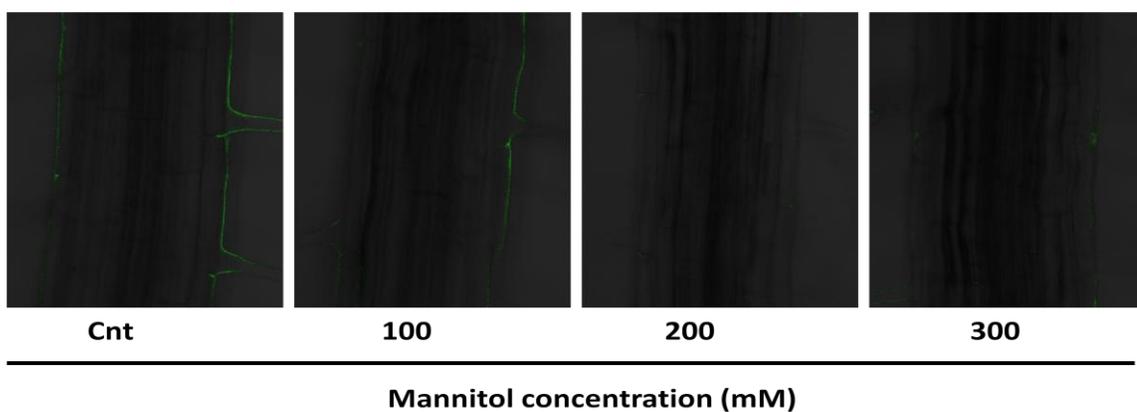


Figure 7.2. Expression of *miR393a::GFP* in response to osmotic stress. Four day old transgenic seedlings carrying *miR393a::GFP* gene construct were treated with mannitol for 18 hrs. Expression of *miR393a::GFP* in roots was detected using Olympus FV1000 confocal microscopy. Images were acquired as described in the legend to figure 7.1.

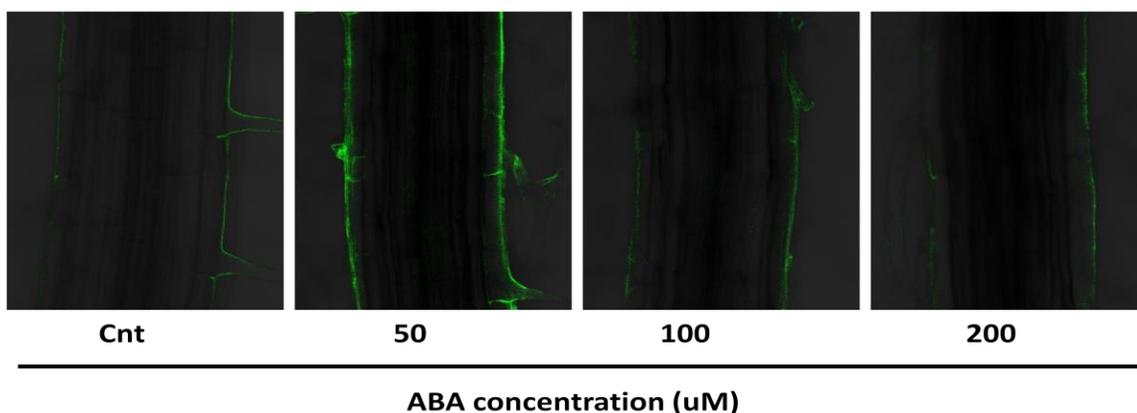


Figure 7.3. Expression of *miR393a::GFP* in response to ABA. Four day old transgenic seedlings carrying *miR393a::GFP* gene construct were treated with ABA for 18 hrs. Expression of *miR393a::GFP* in roots was detected using Olympus FV1000 confocal microscopy. Images were acquired as described in the legend to figure 7.1.

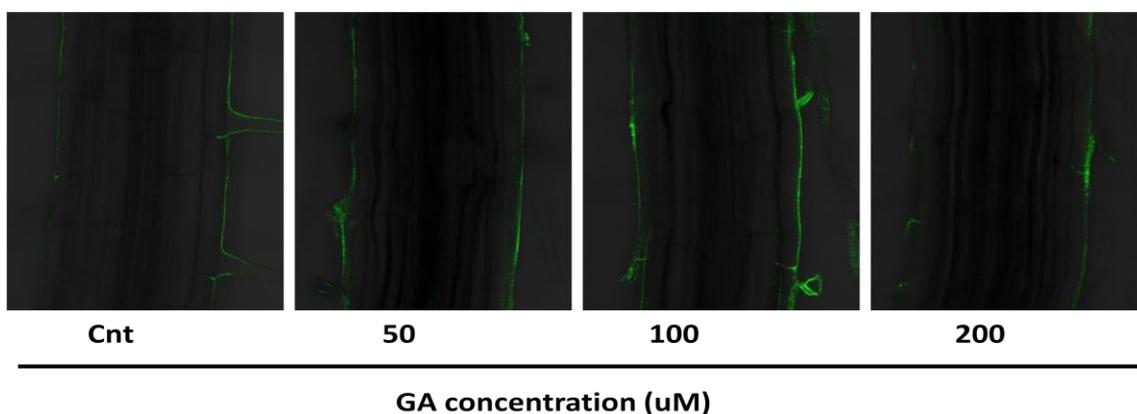


Figure 7.4. Expression of *miR393a::GFP* in response to GA. Four day old transgenic seedlings carrying *miR393a::GFP* gene construct were treated with GA for 18 hrs. Expression of *miR393a::GFP* in roots was detected using Olympus FV1000 confocal microscopy. Images were acquired as described in the legend to figure 7.1.

Expression of *miR393b* is modulated by salinity, osmotic stress, ABA and GA

Unlike *miR393a::GFP*, *miR393b::GFP* expression is mainly seen in the central vascular region of the root and shows dramatic increase in its expression in response to all the treatments. Level of *miR393b::GFP* is greatly induced by salinity stress (Figure 8.1). Also the expression domain expands to periphery of the root in response to high salinity. Mannitol also induces the expression of *miR393b::GFP*, but the expression is mainly restricted to the vascular region (Figure 8.2). Treatment with ABA and GA also induces the expression of *miR393b::GFP* in a similar manner to NaCl treatment (Figure 8.3 and 8.4); however at very high concentrations of ABA the expression is down-regulated.

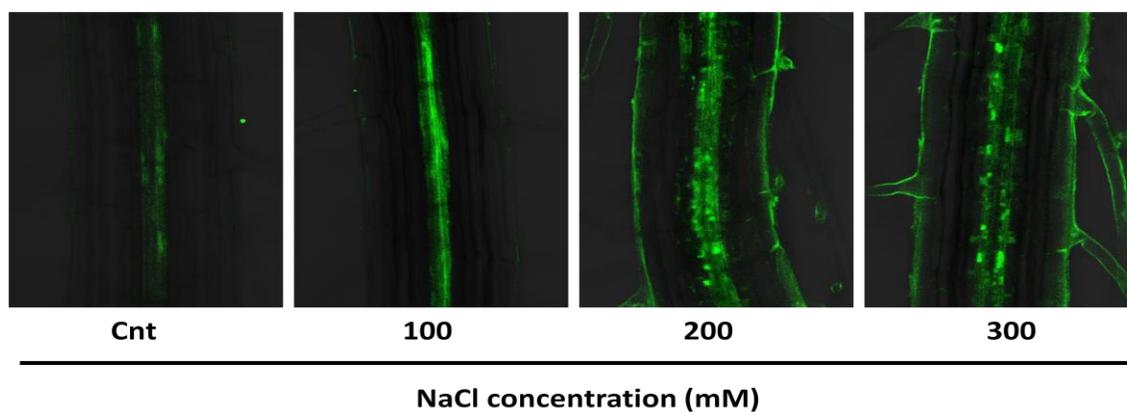


Figure 8.1. Expression of *miR393b::GFP* in response to salinity stress. Four day old transgenic seedlings carrying *miR393b::GFP* gene construct were treated with NaCl for 18 hrs. Expression of *miR393b::GFP* in roots was detected using Olympus FV1000 confocal microscope. Images were acquired as described in the legend to figure 7.1.

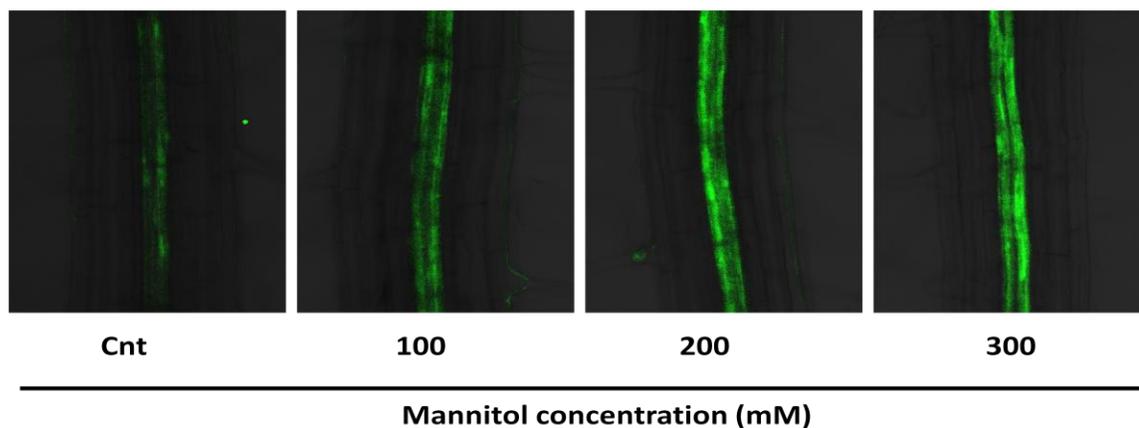


Figure 8.2. Expression of *miR393b::GFP* in response to osmotic stress. Four day old transgenic seedlings carrying *miR393b::GFP* gene construct were treated with mannitol for 18 hrs. Expression of *miR393b::GFP* in roots was detected using Olympus FV1000 confocal microscope. Images were acquired as described in the legend to figure 7.1.

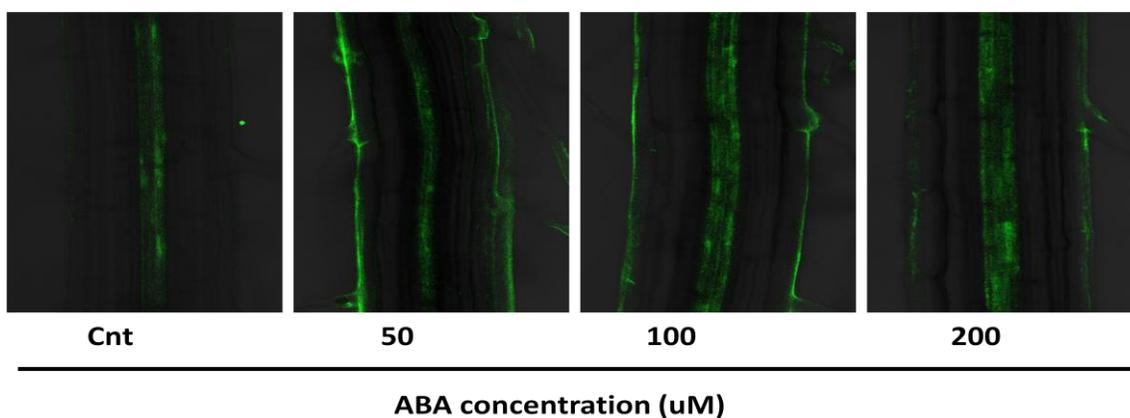


Figure 8.3. Expression of *miR393b::GFP* in response to ABA. Four day old transgenic seedlings carrying *miR393b::GFP* gene construct were treated with ABA for 18 hrs. Expression of *miR393b::GFP* in roots was detected using Olympus FV1000 confocal microscope. Images were acquired as described in the legend to figure 7.1.

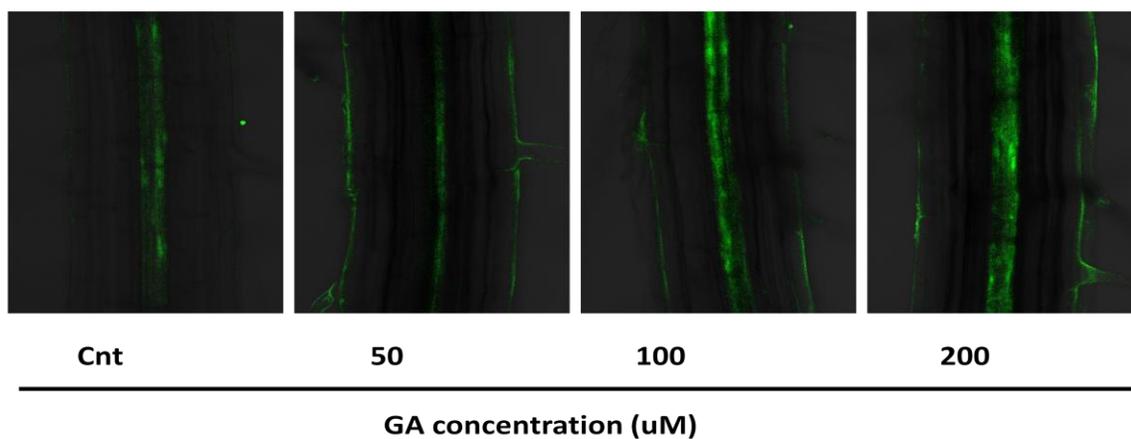


Figure 8.4. Expression of *miR393b::GFP* in response to GA. Four day old transgenic seedlings carrying *miR393b::GFP* gene construct were treated with GA for 18 hrs. Expression of *miR393b::GFP* in roots was detected using Olympus FV1000 confocal microscope. Images were acquired as described in the legend to figure 7.1.

Germination assays

To confirm the effects of salinity, osmotic stress, ABA and GA on plant development, wild type of two different ecotypes (Col-0 and Ws) and auxin receptor mutant seedlings were tested on control and treatment media. In this experiment, the ability to produce green cotyledons when grown under different conditions was considered as evidence of the resistance of the seedling. Therefore, percentage green cotyledons were calculated for comparison. Mutants used in this experiment were from two different ecotypes: *tir1-9*, *afb1-1*, *afb2-1*, *afb3-1* and *afb5-1* belong to Ws while *tir1-1*, *afb2-5*, *afb1-3*, *afb3-4* belong to Col-0.

Auxin receptor mutants are resistant to salinity stress

When wild type (Col-0 or Ws), single, double, triple or quadruple auxin receptor mutants are grown on ATS, all of them produce green cotyledons, even though quadruple mutant shows slower growth than others (Figure 9.1). However, on the ATS medium containing 160 mM NaCl, mutants show various levels of resistance while wild type seedlings are sensitive to NaCl (Figure 9.1 and 9.2). *tir1-9*, *afb1-1*, *afb2-1* and *afb3-1* show resistance to salinity stress when compared with Ws wild type (Figure 9.2). Among them *tir1-9* shows the highest resistance, *afb2-1* and *afb3-1* have moderate resistance, *afb1-1* has the least resistance, while distantly related *afb5-1* does not show resistance at all. The *afb2-1/afb3-1* double mutant is more resistant to salinity compared to either of the single mutants. Similarly, the *afb1-1/afb2-1/afb3-1* triple mutant shows the highest resistance compared to all other mutants. However, the *afb1-1/afb3-1* double mutant shows less resistance to salinity than respective single mutants. While some double

mutants show various levels of resistance, their effects cannot be properly evaluated as two mutants are from two genetic backgrounds. However, in general, successive addition of auxin receptor mutants enhances the salinity resistance even though the quadruple mutant is sensitive to salinity (Figure 9.1 and 9.2).

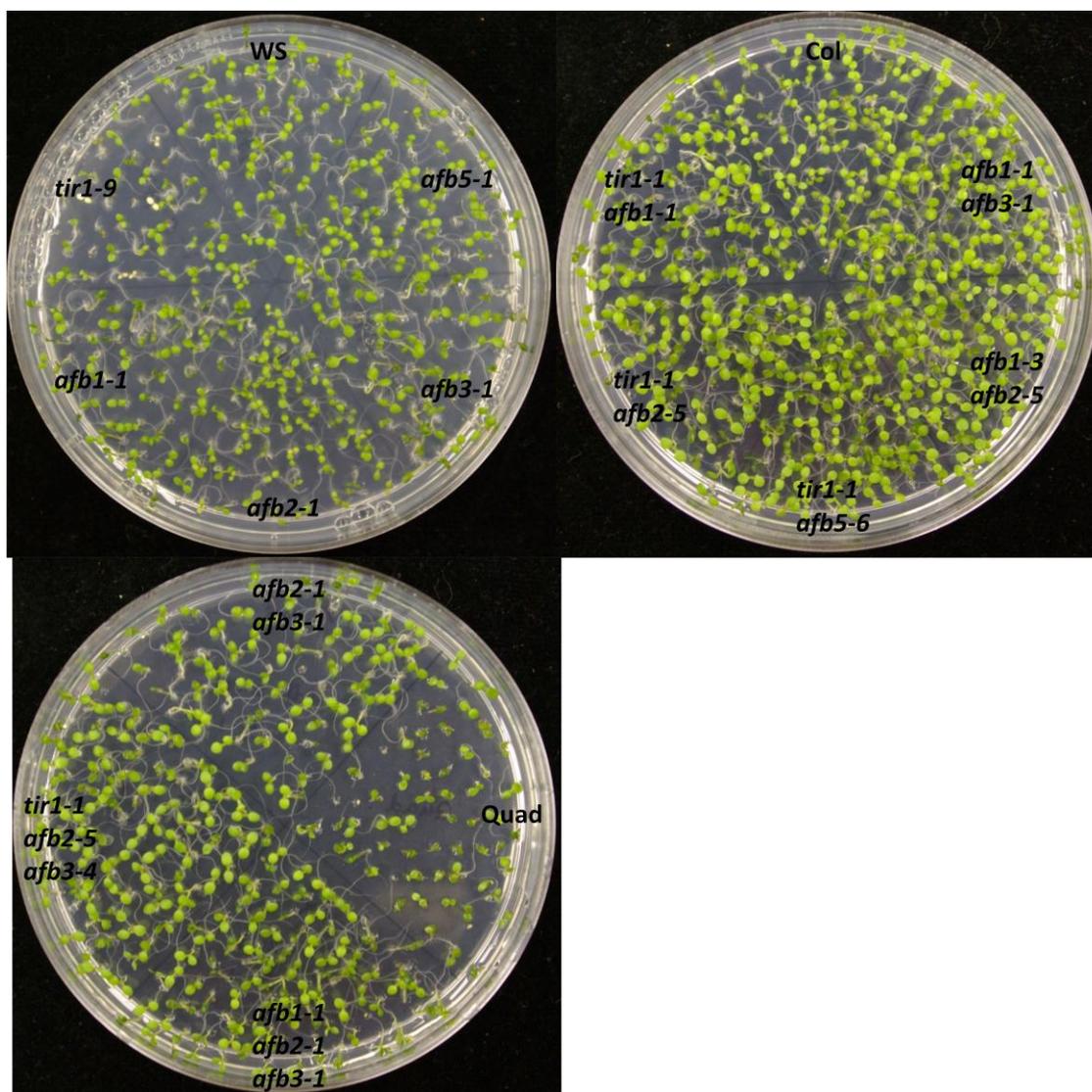
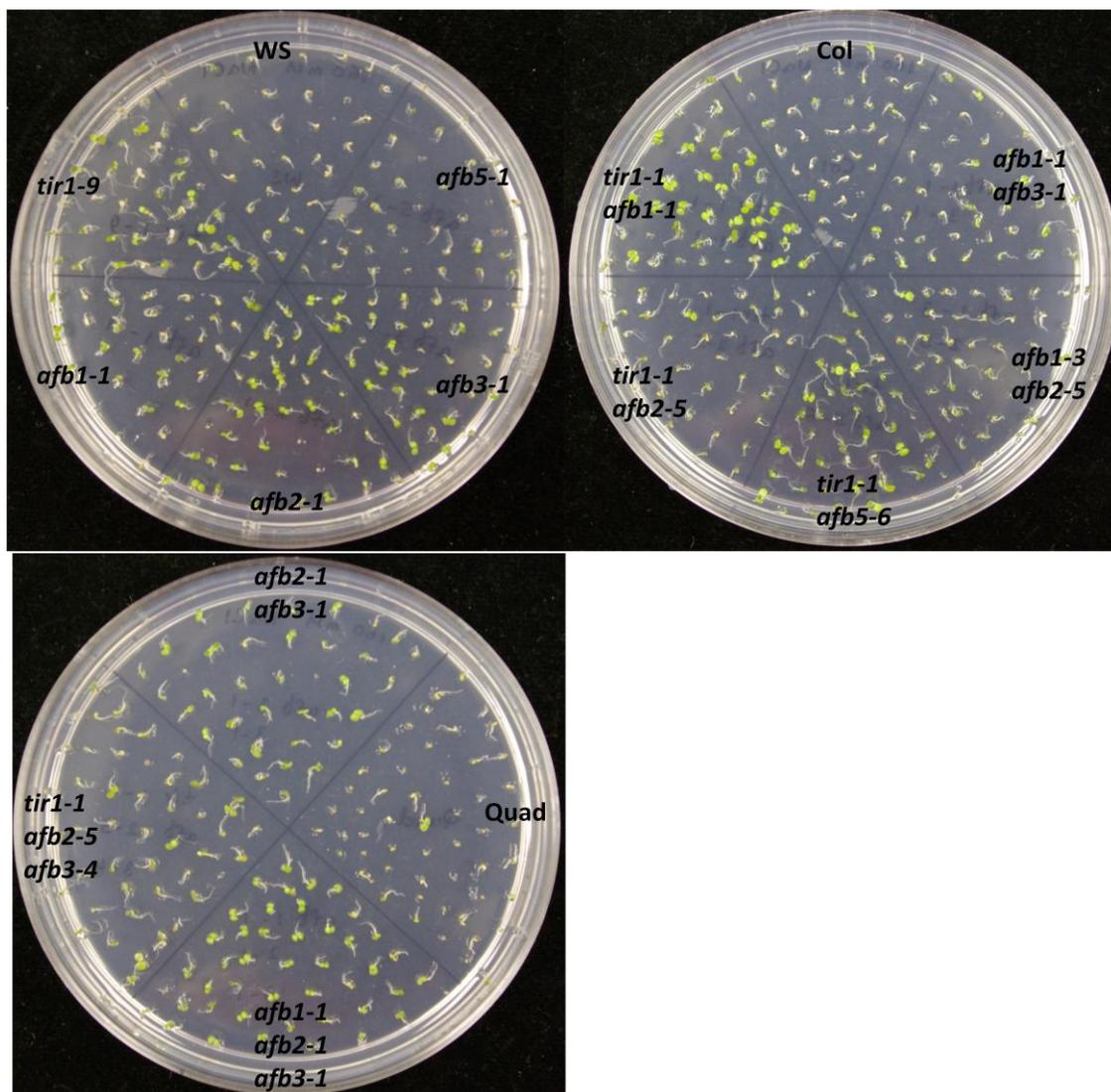


Figure 9.1. Mutants of auxin receptor family grown on ATS (control) media. Seeds were sterilized with 40% bleach and plated them on ATS media. Stratified seeds for two days at 4°C were grown at 21°C for 7 days under continuous illumination.

(a)



(b)

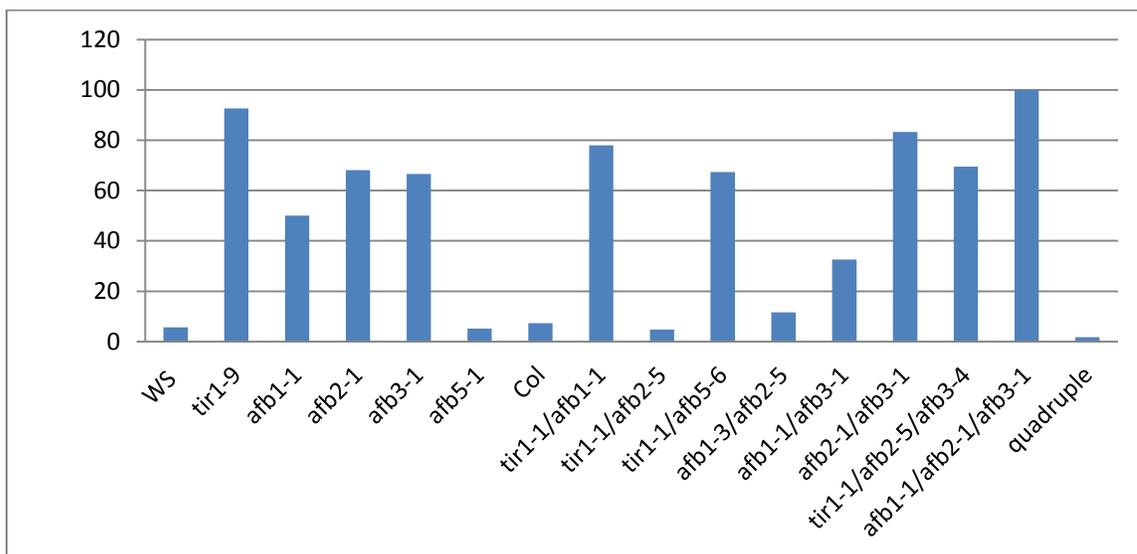
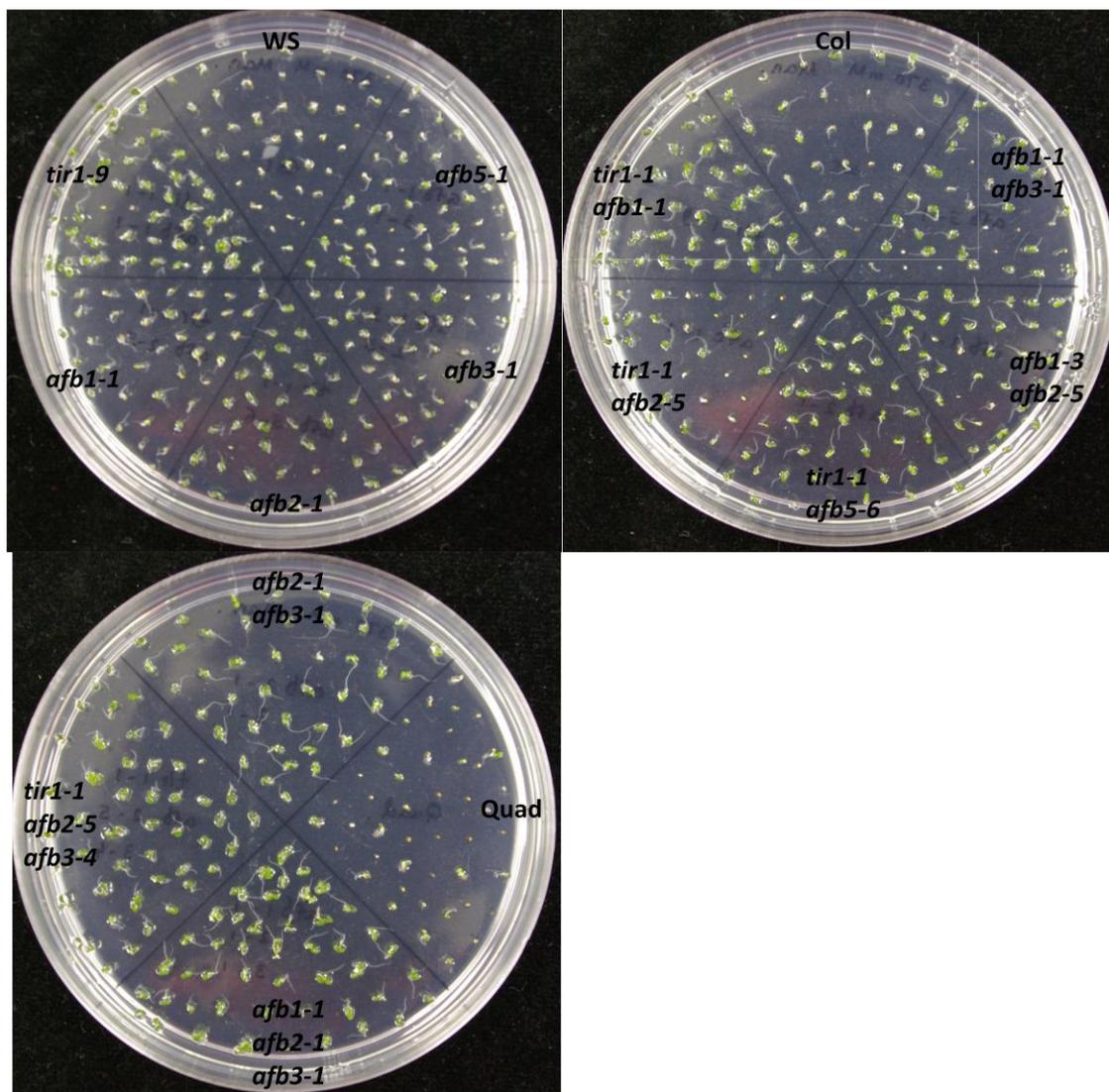


Figure 9.2. (a) Mutants of auxin receptor family grown on ATS media containing 160 mM NaCl. Seeds were sterilized with 40% bleach and then plated on ATS media containing NaCl. Stratified seeds for two days at 4°C were grown at 21°C for 7 days under continuous illumination. (b) Percentage of seedlings with green cotyledons consequent to salinity stress. Seedlings producing green cotyledons were counted after 7 days of growth and calculated as a percentage for each mutant.

tir1, *afb2* and *afb3* are resistant to osmotic stress

In the presence of high concentration of mannitol, the *Ws* ecotype show moderate resistance compared to *Col-0* ecotype. The mutants *tir1-9*, *afb2-1* and *afb3-1* show higher level of resistance to osmotic stress compared to their wild type (*Ws*) while *afb1-1* does not show any resistance. The *afb2-1/afb3-1* double mutant and *afb1-1/afb2-1/afb3-1* triple mutant have the highest resistance to mannitol. Distantly related *afb5-1* also shows resistance to osmotic stress (Figure 9.3 a and b).

(a)



(b)

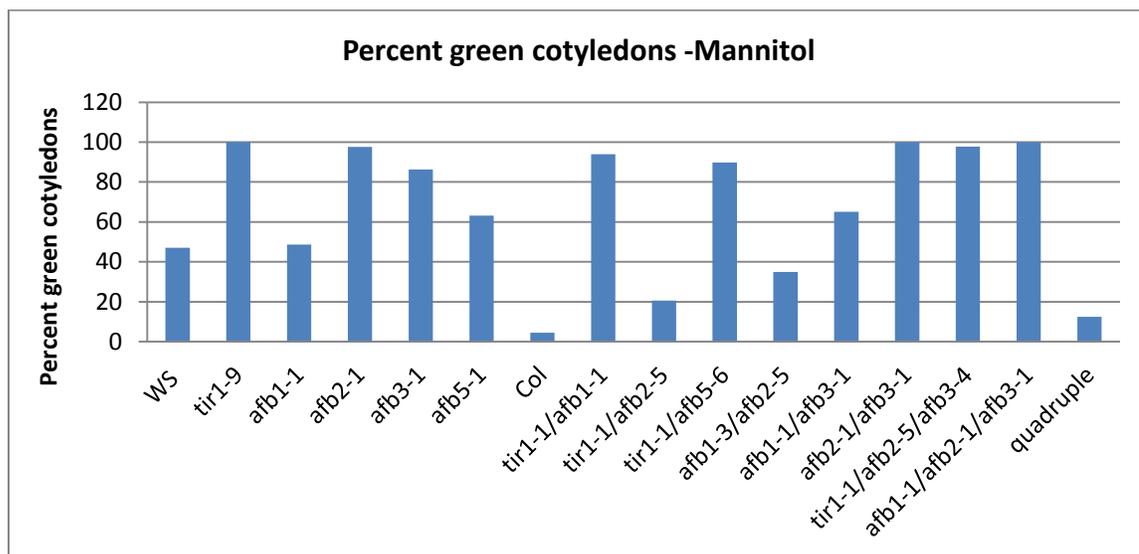


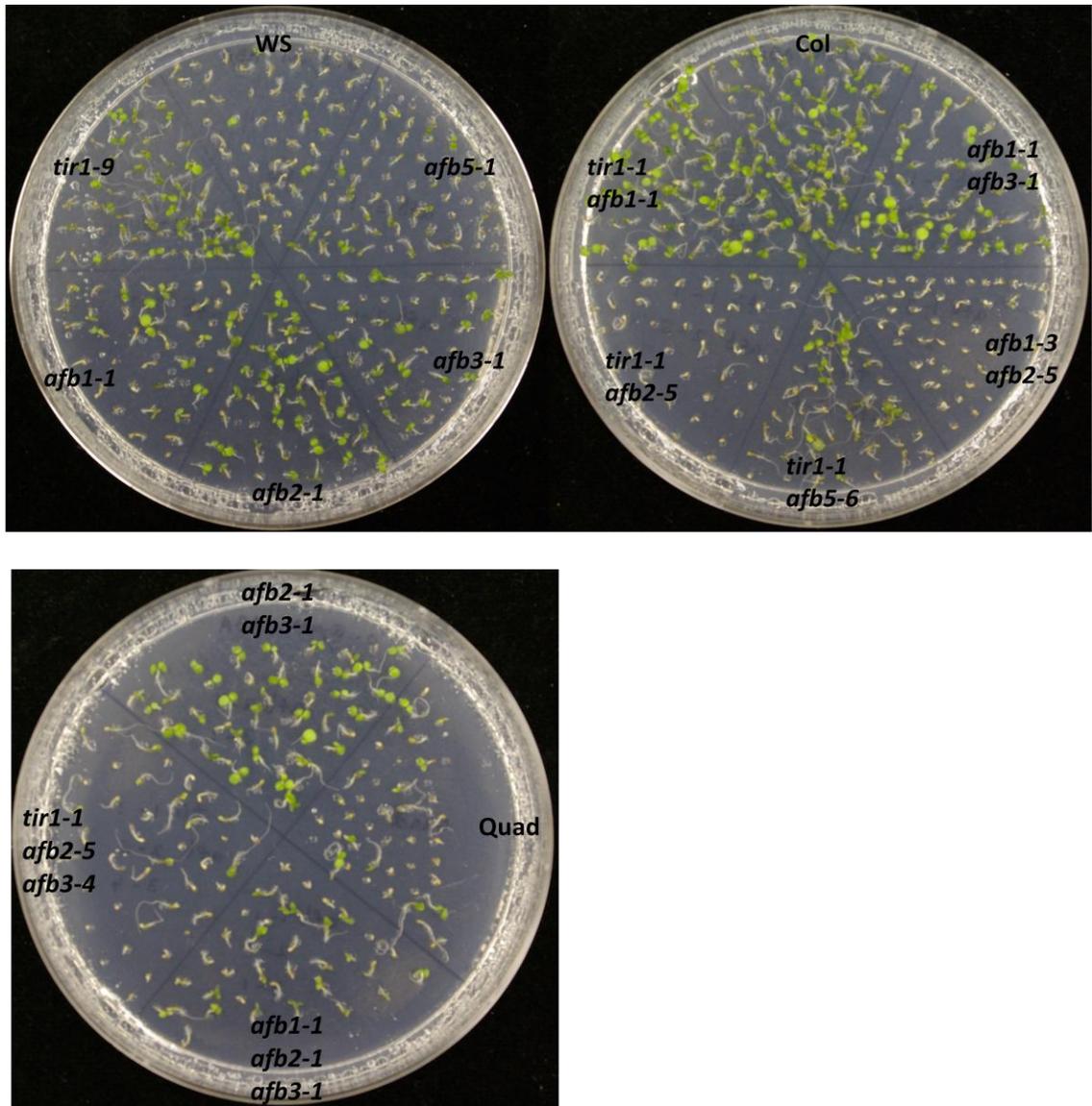
Figure 9.3. (a) Mutants of auxin receptor family grown on ATS media containing 370 mM mannitol. Seeds were sterilized with 40% bleach and then plated on ATS media containing mannitol. Stratified seeds for two days at 4°C were grown at 21°C for 7 days under continuous illumination. (b) Percentage of seedlings with green cotyledons in response to osmotic stress. Seedlings producing green cotyledons were counted after 7 days of growth and calculated as a percentage for each mutant.

Auxin receptor mutants are resistant to ABA

While the wild type *Ws* ecotype is sensitive to exogenous ABA, wild type of *Col-0* ecotype shows considerable resistance (Figure 8.4 a and b). All the receptor family mutants in *Ws* ecotype are resistant to ABA, and *afb2-1* shows the highest resistance. *tir1-9* and *afb3-1* have moderate resistance levels while *afb1-1* and *afb5-1* show low resistance. *afb1-1/afb3-1* and *afb2-1/afb3-1* double mutants also show high resistance to ABA. However, *afb1-1/afb2-1/afb3-1* triple mutant shows the least resistance to ABA

compared to all above mutants. Interestingly, higher order mutants in Col-0 ecotype show sensitivity to ABA compared to its wild type while higher order mutants in Ws ecotype are more resistant to ABA compared to its wild type (Figure 8.4 b).

(a)



(b)

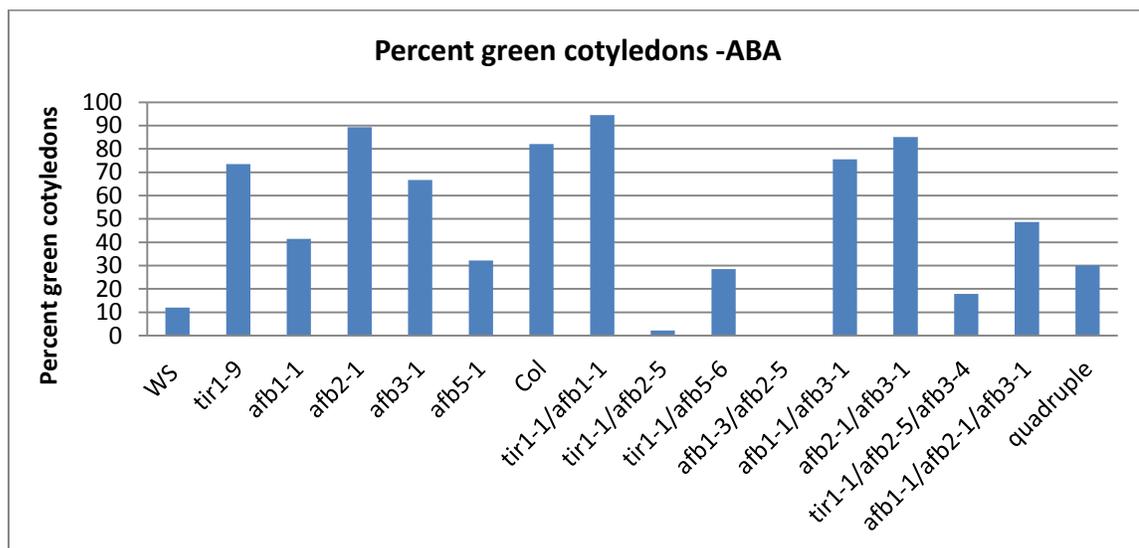
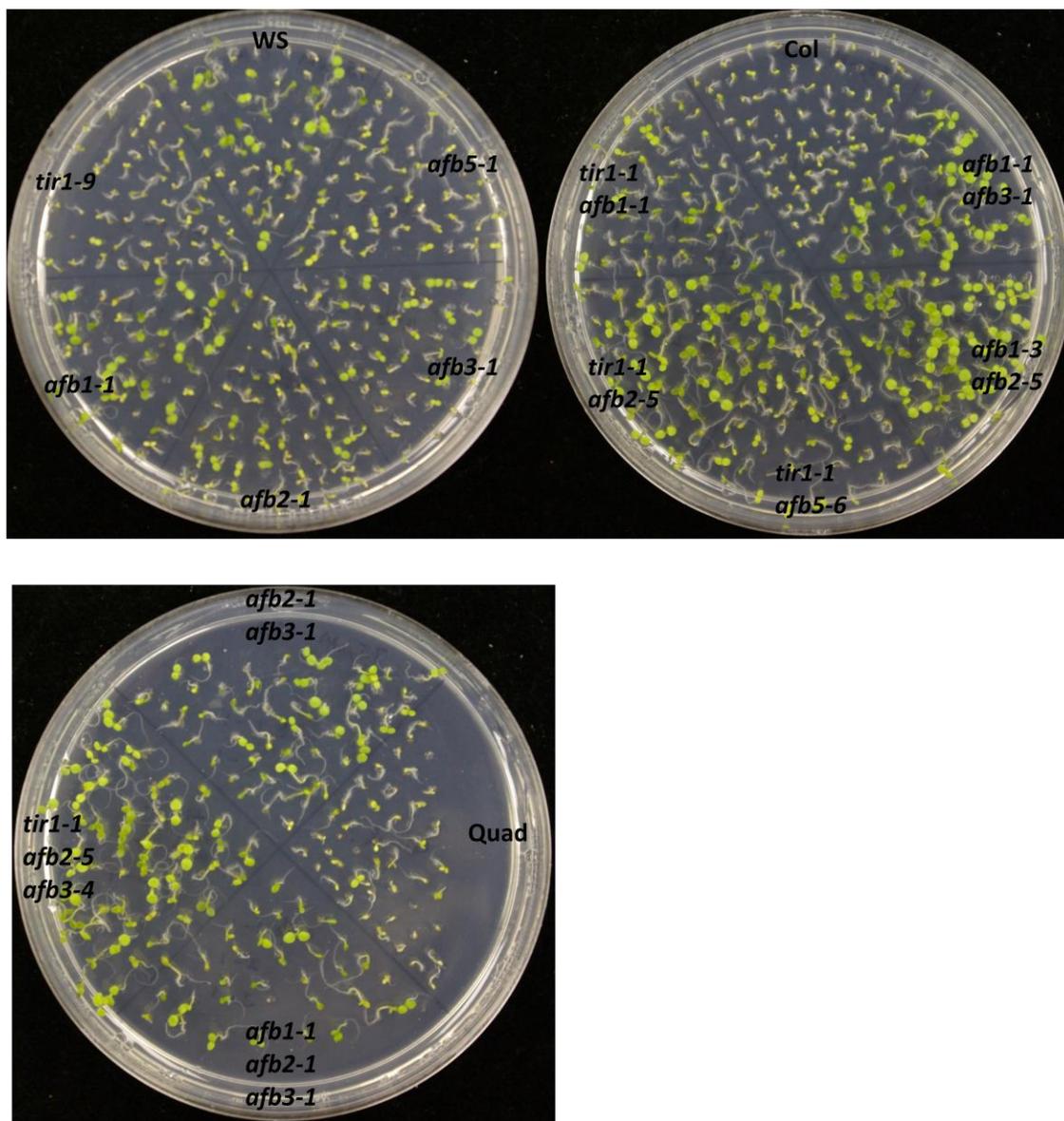


Figure 9.4. (a) Mutants of auxin receptor family grown on ATS media containing 0.5 μM ABA. Seeds were sterilized with 40% bleach and then plated on ATS media containing ABA. Stratified seeds for two days at 4°C were grown at 21°C for 7 days under continuous illumination. (b) Percentage of seedlings with green cotyledons in response to ABA. Seedlings producing green cotyledons were counted after 7 days of growth and calculated as a percentage for each mutant.

afb1 is resistant to GA while other auxin receptor mutants are sensitive

Ws shows comparatively higher resistance than Col-0 to exogenous GA (Figure 9.5a,b). *tir1-9*, *afb2-1* and *afb5-1* are relatively sensitive to GA compared to respective Ws wild type. Only *afb1-1* shows higher resistance to 70 μM GA. However, higher order mutants *afb1-1/afb3-1*, *afb2-1/afb3-1* double mutants and *afb1-1/afb2-1/afb3-1* triple mutant show very high resistance to GA.

(a)



(b)

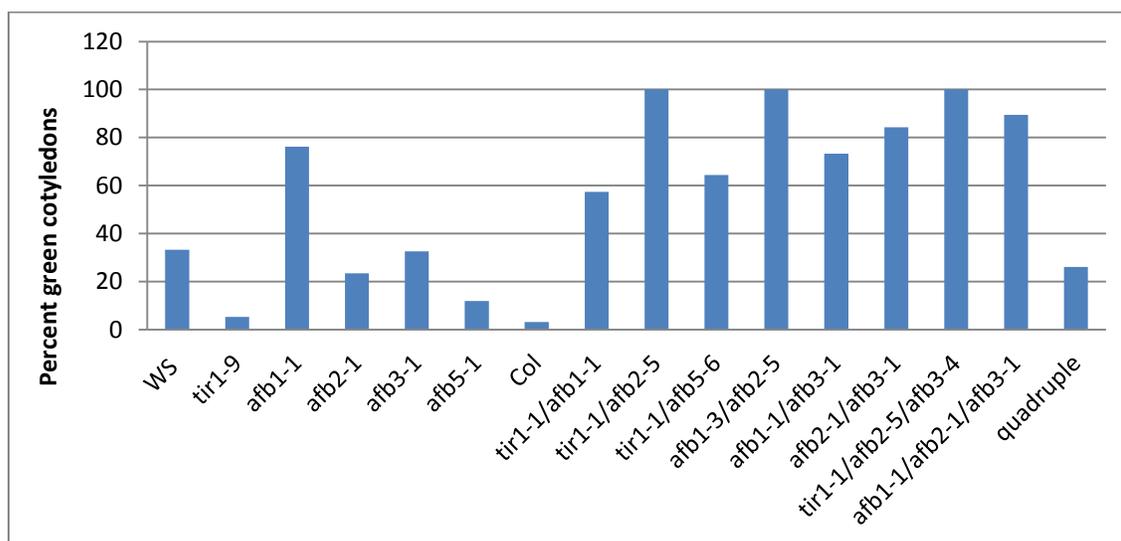
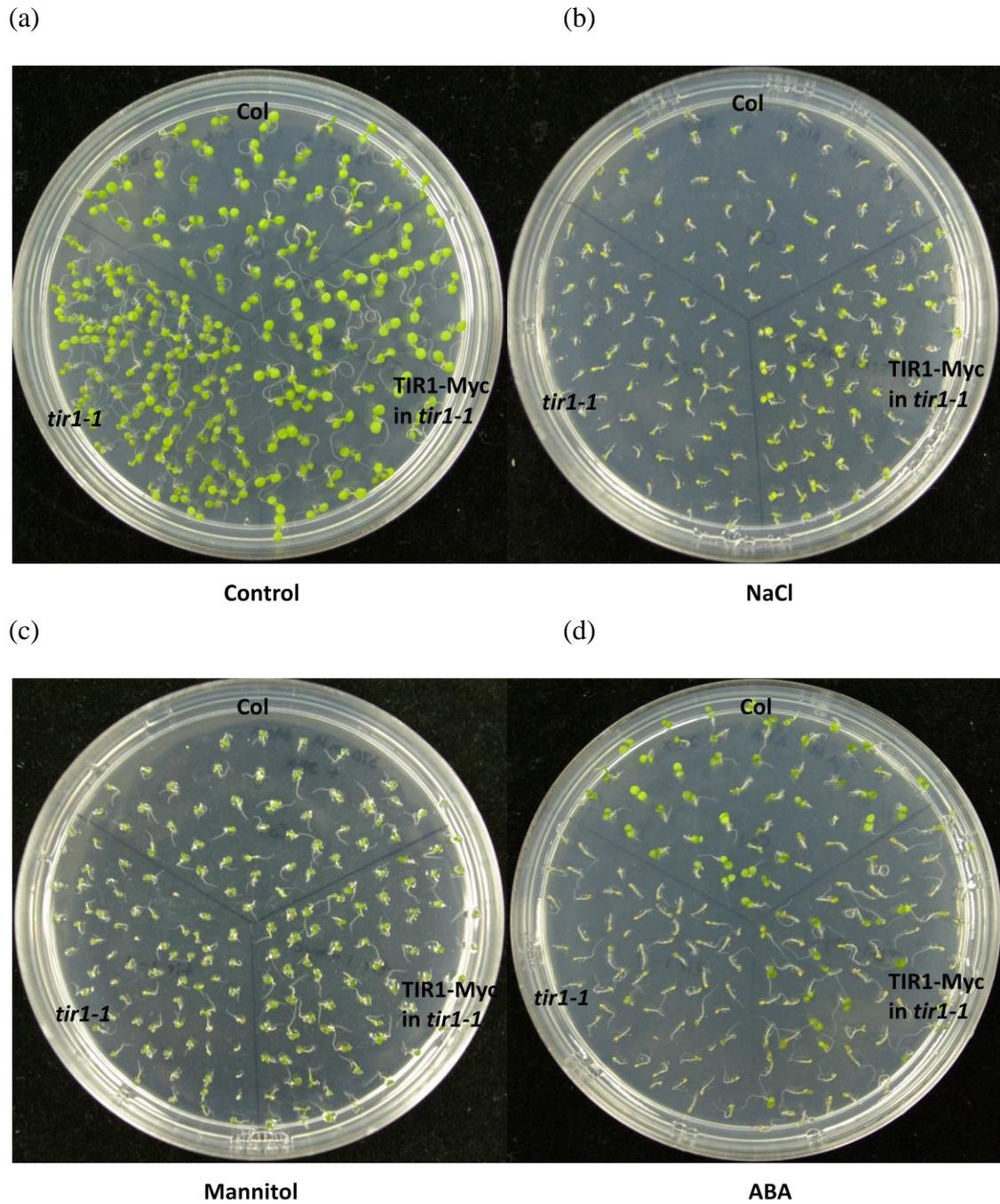


Figure 9.5. (a) Mutants of auxin receptor family grown on ATS media containing 70 μ M GA. Seeds were sterilized with 40% bleach and then plated on ATS media containing GA. Stratified seeds for two days at 4°C were grown at 21°C for 7 days under continuous illumination. (b) Percentage seedlings with green cotyledons in response to GA. Seedlings producing green cotyledons were counted after 7 days of growth and calculated as a percentage for each mutant.

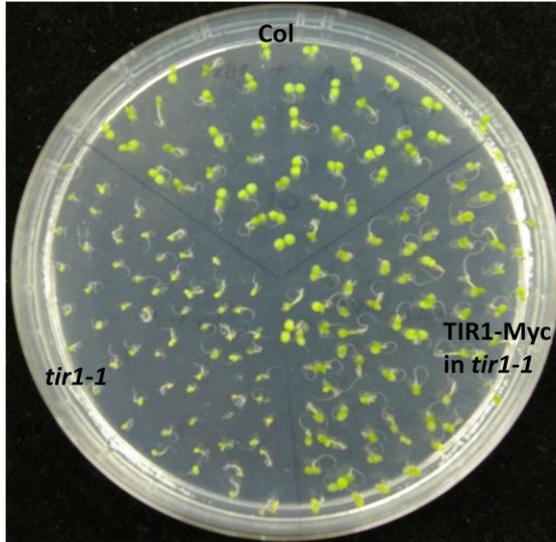
Over-expression of TIR1 complements *tir1-1* phenotypes in response to salinity, osmotic stress, ABA and GA

As *tir1-1* shows defective phenotype in response to salinity, osmotic stress, ABA and GA, a line that is over-expressing TIR1 in *tir1-1* background (*TIR1-Myc / TIR1 OX*) was also tested under these stress conditions. Unlike the *tir1-9* in *Ws* background (Figure 9.2-4), *tir1-1* in *Col-0* background shows hypersensitivity to NaCl, mannitol, ABA and

GA (Figure 10.1). Over-expression of TIR1 in *tir1-1* background completely recovers the sensitivity in response to above conditions.



(e)



(f)

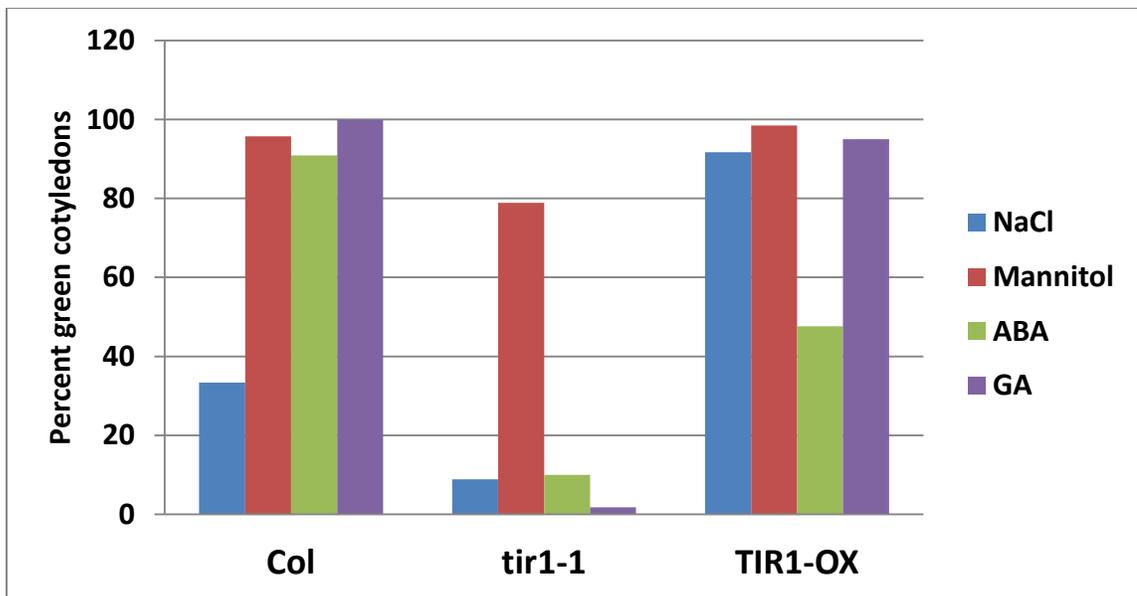


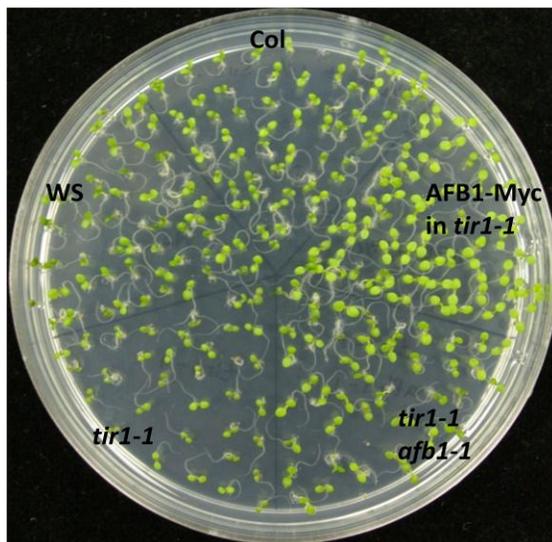
Figure 10.1. Recovery of *tir1-1* phenotype in response to salinity, osmotic stress, ABA and GA by over-expression of TIR1. (a) Normal growth of wild type (Col-0), *tir1-1*, *TIR1-OX* (*TIR1-Myc*) lines on ATS (control) medium. (b) Complementation of *tir1-1* by *TIR1-OX* in response to salinity stress. Seedlings were grown on ATS + 160 mM NaCl.

(c) Complementation of *tir1-1* by *TIR1-OX* in response to osmotic stress. Seedlings were grown on ATS + 370 mM mannitol. (d) Complementation of *tir1-1* by *TIR1-OX* in response to high concentration of ABA. Seedlings were grown on ATS + 0.5 μ M ABA. (e) Complementation of *tir1-1* by *TIR1-OX* in response to high concentration of GA. Seedlings were grown on ATS + 50 μ M GA. (f) Percentage of seedlings with green cotyledons produced in response to NaCl, mannitol, ABA and GA. Seeds were sterilized with 40% bleach and plated on treatment media. Stratified seeds maintained for two days at 4°C were then grown at 21°C for 7 days under continuous illumination. Seedlings with green cotyledons were counted after 7 days of growth, and percentage was calculated.

AFB1 rescues *tir1-1* phenotype in response to salinity, osmotic stress, ABA and GA

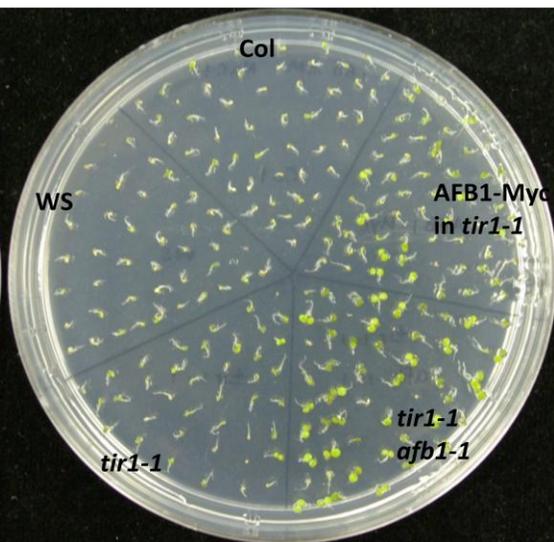
As the *afb1-1* acts differently compare to the other mutants, its ability to recover *tir1-1* was tested using a line that over-expresses *AFB1* in *tir1-1* background (*AFB1-Myc/AFB1 OX*). In response to NaCl and mannitol, it not only recovers *tir1-1* sensitivity, but also confers resistance (Figure 10.2). *AFB1* over expression also recovers ABA and GA sensitivity of *tir1-1* (Figure 10.2).

(a)



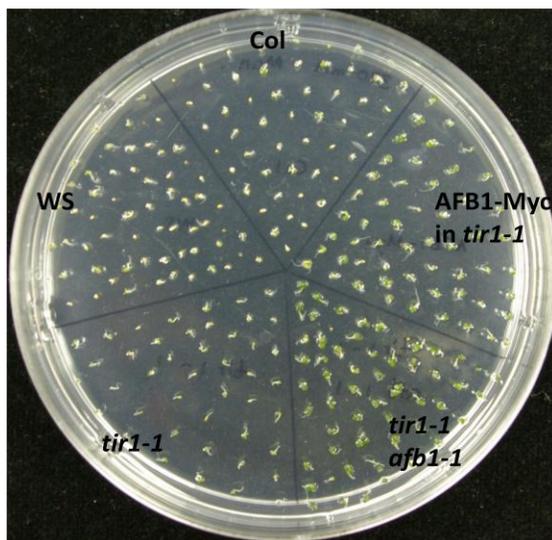
Control

(b)



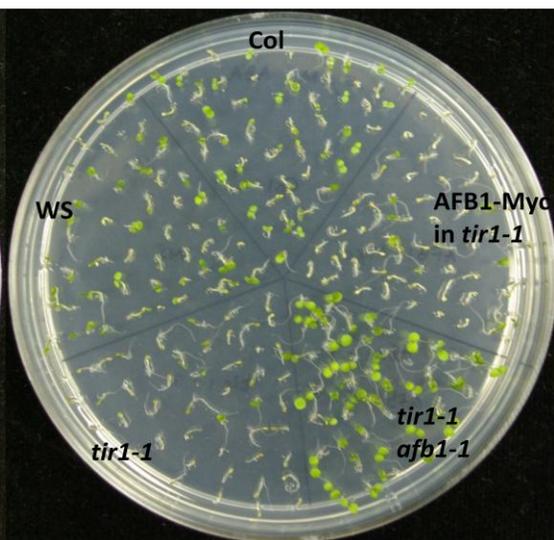
NaCl

(c)



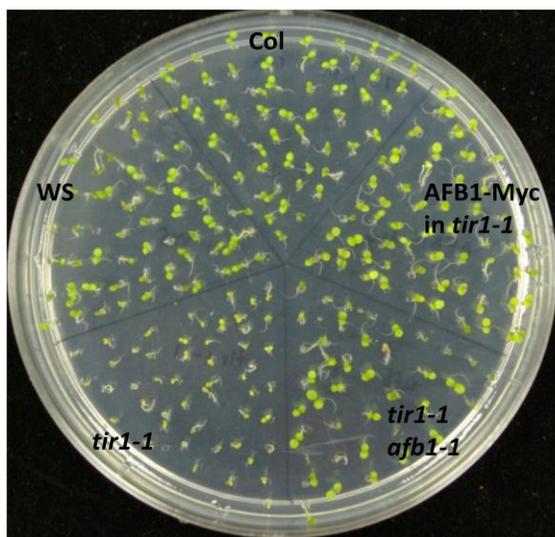
Mannitol

(d)



ABA

(e)



(f)

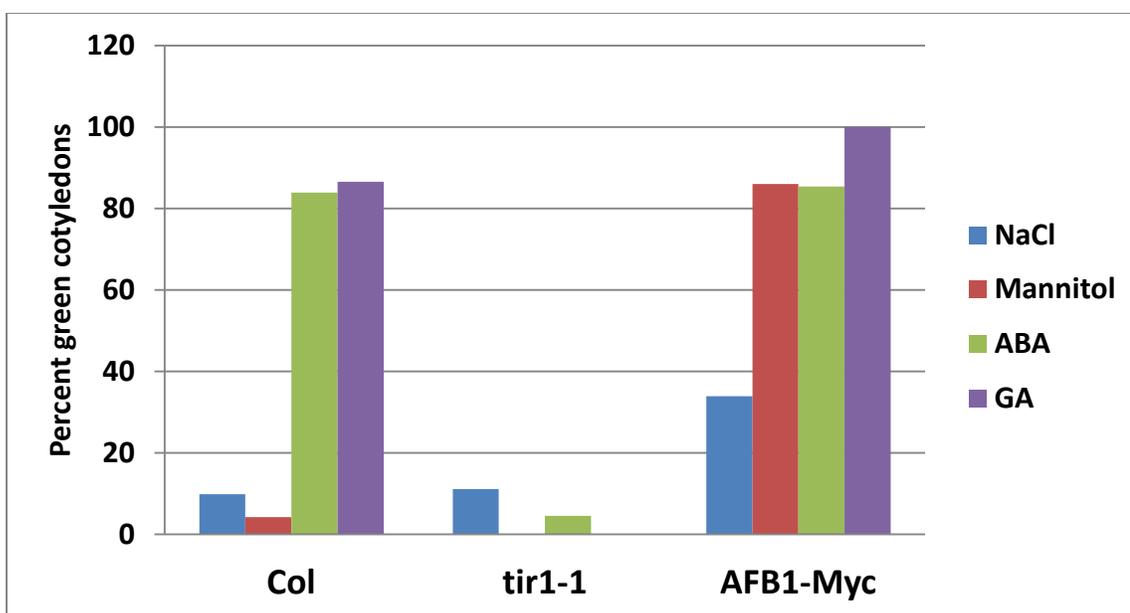


Figure 10.2. Recovery of *afb1-1* phenotype in response to ABA, GA, salinity and osmotic stress by over-expression of *AFB1*. (a) Normal growth of wild type (Col-0), *afb1-1*, *AFB1-OX* in ATS (control) media. (b) Complementation of *tir1-1* by *AFB1-OX* in response to salinity stress. Seedlings were grown on ATS + 160 mM NaCl. (c) Complementation of *tir1-1* by *AFB1-OX* in response to osmotic stress. Seedlings were

grown on ATS+ 370 mM mannitol. (d) Complementation of *tir1-1* by *AFBI-OX* in response to high concentration of ABA. Seedlings were grown on ATS + 0.5 μ M ABA. (e) Complementation of *tir1-1* by *AFBI-OX* in response to high concentration of GA. Seedlings were grown on ATS + 50 μ M GA. (f) Percentage seedlings with green cotyledons produced in response to NaCl, mannitol, ABA and GA. Seeds were sterilized with 40% bleach and plated them on treatment media. Stratified seeds for two days at 4°C were grown at 21°C for 7 days under continuous illumination. Seedlings with green cotyledons were counted after 7 days of growth and percentage was calculated.

Auxin receptor genes contain putative *cis* regulatory elements responsive to osmotic stress, ABA and GA

Analyses of promoter sequences of four auxin receptor genes have revealed the presence of putative *cis*-regulatory elements common to stress and hormonal responses. An ABRE-like sequence is located 141 bp upstream of transcription starting point (TSP) of *TIR1*. Promoter of *AFBI* also contains ABRE like sequence 993 bp upstream of TSP. However, its e-value is higher than that of the ABRE present in *TIR1*. Interestingly, all the auxin receptor genes contain AtMYC2 BS RD22 sequences. *AFBI* has four such sequences while others contain one. All the *AFBs* contain AtMYB2 BS RD22 sequences. Only *TIR1* contains DRE core sequence 262 bp upstream of TSP. All auxin receptor genes except *AFB3* contain Gibberellic acid responsive elements (GARE) in duplicate.

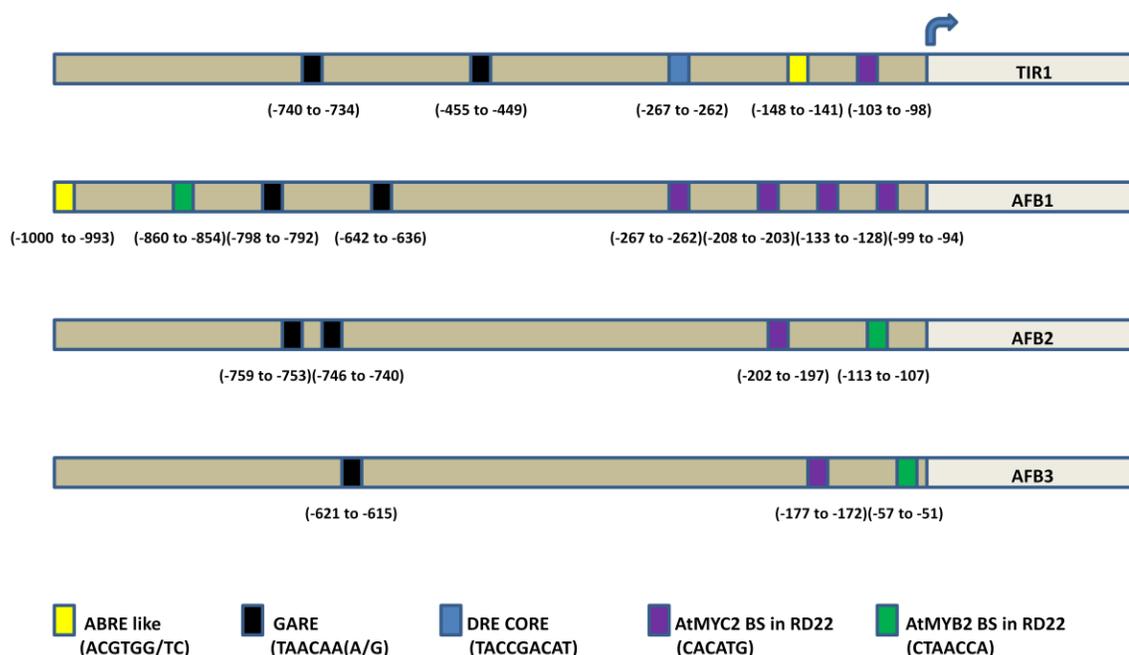


Figure 11. Putative *cis*-regulatory elements found in auxin receptor family genes. The promoter regions (1000 bp upstream of ATG) of *TIR1*, *AFB1*, *AFB2* and *AFB3* genes were analyzed using ATHENA, ATHAMAP, PROMOTER and AGRIS web based bioinformatics tools. Putative regulatory elements were identified depending on the threshold e-value and the consensus sequence.

TIR1 promoter contains a bona fide ABRE

Putative ABRE like sequence in *TIR1* promoter was altered by changing ACGTGTC into ACGTCTC (Hattory et al., 2002). A promoter containing the altered ABRE sequence was cloned in front of the GUS coding sequence to generate the *TIR1*_{mABRE}::GUS construct and transformed into *Arabidopsis* wild type Col-0. Homozygous plants carrying *TIR1*_{mABRE}::GUS were used for further experiments. Several lines of *TIR1*_{mABRE}::GUS having different levels of expressions were treated with ABA

and compared with *TIR1::GUS* expression pattern. Interestingly, ABA does not induce the mutated *TIR1_{mABRE}::GUS* expression. This observation suggests that putative ABRE sequence found in *TIR1* promoter is a true ABRE and it regulates the *TIR1* expression in response to ABA.

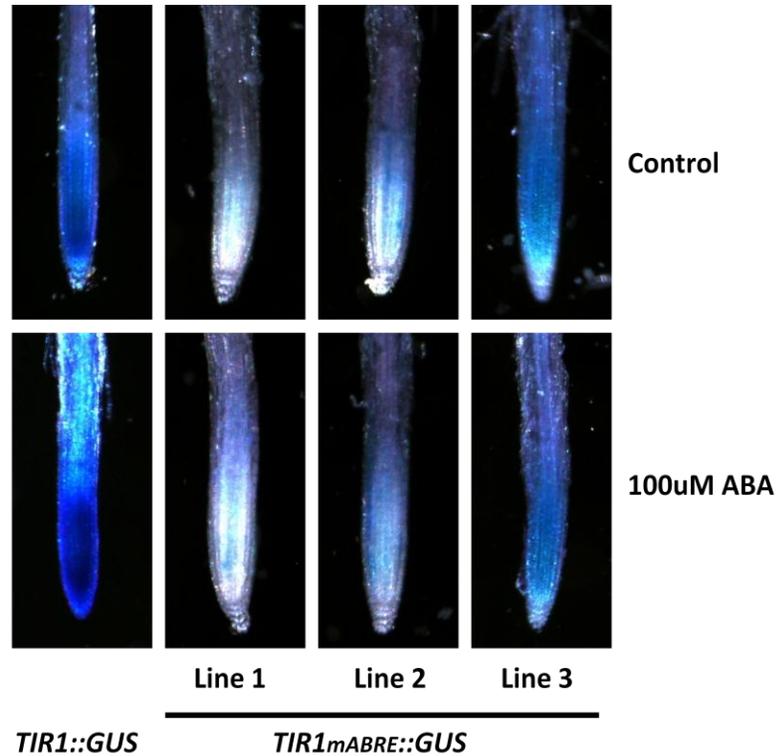


Figure 12. ABA fails to induce *TIR1_{mABRE}::GUS* expression. Roots of four day old seedlings carrying *TIR1::GUS* or *TIR1_{mABRE}::GUS* were stained for GUS activity. Three independent lines of *TIR1_{mABRE}::GUS* having different levels of expression were used in the experiment.

CHAPTER IV

DISCUSSION

As the major plant hormone, auxin regulates many aspects of plant growth and development. Recent studies indicate that the auxin response pathway interacts with other plant hormone signaling pathways in multiple ways (Depuydt et al., 2011). Therefore, the final outcome of growth responses is due to the crosstalk between several plant hormones. As plants are normally exposed to stress conditions such as salinity and osmotic stress, plant hormone signaling pathways should be fine-tuned to cope with changing environmental conditions. Previous studies have shown that ABA and GA signaling are involved in regulating auxin signaling and vice versa (Bjorklund et al., 2007, Sun et al., 2005). Reduced polar auxin transport affects GA biosynthesis as well as GA-induced degradation of DELLA proteins. Thus, auxin affects both GA biosynthesis and GA signaling (Fu et al., 2003). ABA alters the expression of auxin transporter genes in response to salinity stress (Yu et al., 2010). Auxin related mutants such as *ibr5-1* show defects in ABA signaling as well, suggesting an interaction between the two hormone signaling pathways (Strader et al., 2008). Salinity and osmotic stresses also have direct effects on auxin signaling. According to Iglesias et al. (2010), auxin receptors TIR1 and

AFB2 are involved in adapting the plant to salinity and oxidative stresses. However, the underlying molecular mechanism is not yet known. According to the current model, auxin interacts with its co-receptors TIR1/AFBs and transcriptional repressor Aux/IAs, mediating ubiquitination and subsequent degradation of Aux/IAs. Removal of Aux/IAs through degradation modulates the transcription of auxin responsive genes. Therefore, it is arguable that if the abundance of auxin receptor proteins is altered, downstream auxin signaling will also be changed.

In this work the expression of auxin receptor family genes was studied in response to salinity, osmotic stress, ABA and GA, using transcriptional and translational reporter gene constructs as major molecular tools. β -glucuronidase (GUS) enzyme was one of the reporter genes used. This enzyme converts its substrates X-Gluc (5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide) into a blue colored product or MUG (4-methylumbelliferyl- β -D-galactopyranoside) into a fluorescent product. X-Gluc was used in histochemical staining, and MUG was used in quantitative GUS assay. In order to study the mRNA level prior to post transcriptional modifications, transcriptional reporter constructs were used where promoters of *TIR1*, *AFB1*, *AFB2* and *AFB3* were fused to GUS reporter gene. Translational reporter constructs used are a fusion of promoter and coding region of the gene of interest to GUS reporter gene. Translational constructs of above genes were used to study the protein levels in response to salinity, osmotic stress, ABA and GA. Another reporter gene used was green fluorescent protein (GFP), the coding sequence for which was fused to promoters of *miR393a* and *miR393b*. These reporters were used to study the expression pattern of *miR393* in response to above

conditions, as it is known to be a negative regulator of auxin receptor genes (Sunkar et al., 2004).

TIR1 expression is regulated by salinity, osmotic stress, ABA and GA

TIR1 transcription is induced by NaCl in both shoots and roots. However, at high concentrations of NaCl, *TIR1* expression is down-regulated in roots but up-regulated in shoots, suggesting differential regulation of its expression within the plant. However, according to the results of *TIR1::TIR1-GUS* translational construct, in both shoots and roots *TIR1* expression is up-regulated by low salinity while down-regulated by high salinity. This observation suggests that *TIR1* transcripts are subjected to tight regulation prior to translation. Nevertheless, translation and stability of the protein may also be affected by high salinity conditions. According to RT-PCR analysis which indicates the endogenous transcript level, *TIR1* is up-regulated comparable with reporter gene expression data at low concentrations; however, the down-regulation seen in reporter gene expression at the highest concentration (300 mM) of NaCl is not evident from RT-PCR data. Therefore, it is possible that translation or the stability of TIR1 protein is affected at high salt conditions.

Since NaCl causes both salinity stress and osmotic stress, seedlings were treated with mannitol to check whether the above effect was from osmotic stress. Osmotic stress does not induce *TIR1::GUS* expression, but it does induce *TIR1::TIR1-GUS* expression. This observation suggests that *cis*-regulatory elements required for osmotic stress induction may be present within the gene. Alternatively, *TIR1-GUS* protein may be stabilized by osmotic stress. However, the induction was much less compared to that of

NaCl, suggesting that salinity, but not osmotic stress, mainly contributes to the induction of *TIR1* transcription. Similar to salinity, high osmotic stress also results in down-regulation of *TIR1* expression. The expression in shoots and roots follows the same pattern in response to osmotic stress. RT-PCR data show a slight increase in *TIR1* transcription, paralleling reporter expression data.

Since salinity and osmotic stress induce *TIR1* expression, and since abscisic acid level and its signaling are also enhanced by above stresses (reviewed by Zhu et al., 2002), *TIR1* expression was examined in the presence of ABA. Similar to salinity and osmotic stresses, ABA up-regulates *TIR1* expression in roots. However, it does not up-regulate *TIR1* expression in the shoot, but rather down-regulates it at higher concentrations. Despite the down-regulation of *TIR1* in the shoot at high concentrations of ABA evident from reporter gene constructs, the RT-PCR data indicate that *TIR1* is up-regulated in response to ABA. These data again suggests the complexity of the regulation of *TIR1* expression within the plant.

As gibberellic acid signaling also interacts with auxin signaling and it acts as an antagonist to ABA in several biological processes (reviewed by Hartweck., 2008), the ability of GA to regulate *TIR1* expression was studied. Interestingly, GA also induces *TIR1* expression. This induction is evident in both reporter gene expression and RT-PCR analysis.

AFBI expression is regulated by hormone and stress factors.

According to reporter gene expression data, *AFBI* follows the same expression pattern as *TIR1* in response to NaCl and mannitol, Both are up-regulated by low

concentrations and down-regulated by high concentrations. Peak expression of *AFB1* was observed at 100 mM concentration while for *TIR1*, peak expression was at 200 mM concentration of NaCl and mannitol. In contrast to reporter gene expression data, RT-PCR indicates that *AFB1* is down-regulated even at low concentrations of NaCl and mannitol. It is possible that the difference between the RT-PCR results and the reporter construct results can be attributed to the fact that only 2000 bps upstream of the *AFB1* gene start site is included as the promoter in the reporter gene. Therefore, additional *cis*-regulatory elements needed for fine tuning transcription may not be within the promoter region used in the construct. The difference may also be due to post transcriptional modifications of *AFB1* that do not affect the *AFB1::AFB1-GUS* transgenic construct, modifications such as *miR393* mediated gene regulation. Interestingly, ABA down-regulates *AFB1* expression based on results with the reporter constructs. This finding was corroborated by RT-PCR results, which clearly show the down-regulation of *AFB1* in response to ABA. In contrast to ABA, gibberellic acid dramatically induces *AFB1* expression as shown in reporter gene expression and RT-PCR results. However, the down-regulation of *AFB1* at high concentrations of GA was not evident in RT-PCR results. The expression pattern in response to all the treatments was similar in both shoots and roots.

AFB2 expression is down-regulated by salinity, osmotic stress and ABA

Salinity, osmotic stress and ABA down-regulate *AFB2* at all concentrations tested according to reporter gene expression as well as RT PCR results. Even though *AFB2* is down-regulated by above treatments, GA induces its expression at low concentrations

and down-regulates it at high concentrations, following a similar pattern as observed with *TIR1* and *AFB1* reporter constructs. These expression patterns are evident in both reporter gene expression as well as RT-PCR data.

AFB3 follows the similar expression pattern as *AFB2* except in response to ABA

Similar to *AFB2*, *AFB3* is also down-regulated by salinity and osmotic stress as shown in reporter gene expression and RT-PCR data. However unlike *AFB2*, the expression of *AFB3* is up-regulated by low concentrations of ABA but down-regulated by high concentrations of ABA as evident from *AFB3::GUS* and *AFB3::AFB3-GUS* expression. This differential regulation is also evident in RT-PCR analysis. In response to GA, *AFB3* is up-regulated at low concentrations and down-regulated at high concentrations as observed with the other genes.

Considering the expression patterns of all four genes, *TIR1* behaves differently from *AFBs* in response to salinity and osmotic stress. However, *TIR1* and *AFB3* show a similar expression pattern in response to ABA. *AFB1* and *AFB2* show a similar expression pattern with each other, which is different from that of *TIR1* and *AFB3* expression. Gibberellic acid regulates the expression of all four genes in a similar fashion. Despite being in the same family, each auxin receptor gene contributes differently to auxin signaling in response to different environmental cues. Low phosphate availability induces *TIR1* expression, but not other *AFBs*' expression (Torres et al., 2008). Also *AFB3*, but not other receptor genes, is involved in changing the root system architecture in response to nitrate (Vidal et al., 2009). Therefore, the observed results in

this study along with previous studies further confirm the complexity of the regulation of auxin receptor genes by different environmental factors.

miR393 expression is enhanced by salinity, ABA and GA

One known post-transcriptional mechanism that regulates gene expression is RNA silencing. Sequence specific mRNA degradation occurs during RNA silencing mediated by 20 to 24 nucleotide RNAs known as microRNAs (miRNAs) and short interfering RNAs (siRNAs). According to previous studies, *TIR1/AFBs* are negatively regulated by *miR393* and siRNAs. In addition, the origin of *miR393* varies depending on the factor that triggers it such as microbes, nitrates or developmental stage (Navarro et al., 2006, Vidal et al., 2010, Ammour et al., 2011). Therefore, the expression of *miR393a* and *miR393b* was studied using transcriptional GFP fusion constructs. In wild type seedlings, expression of *miR393a::GFP* is considerably lower than that of *miR393b::GFP* (Figure 6.1 and 7.1). Additionally, the expression of *miR393a::GFP* is confined to periphery of the root while the expression of *miR393b::GFP* is mainly seen in the central vascular region of the root. Salinity induces the expression of both *miR393a::GFP* and *miR393b::GFP*, and the induction is very prominent with *miR393b::GFP* (Figure 6.1 and 7.1). Moreover, *miR393b::GFP* expression that is normally confined to the central vascular region in untreated seedlings can be seen in the periphery of the root when treated with NaCl. Conversely, mannitol treatment down-regulates *miR393a::GFP* expression, but induces *miR393b::GFP* expression. ABA and GA show similar effects on the expression of both *miR393a::GFP* and *miR393b::GFP*. These hormones up-regulate the expression of these genes at low concentrations but

down-regulate the expression at higher concentrations (Figures 7.3, 7.4, 8.3 & 8.4).

These results suggest the possible involvement of *miR393a* and *miR393b* in regulation of auxin receptor genes in response to NaCl, osmotic stress, ABA and GA.

It is important to notice that *miR393* silencing of auxin receptor genes is a tightly regulated process. According to Navarro et al. (2006), *miR393a* derived *miR393* cleaves *TIR1*, *AFB2* and *AFB3* transcripts but not *AFB1* transcripts in response to bacterial flagellin²². However, only *AFB3* but not *TIR1*, *AFB1* or *AFB2* transcripts are cleaved by *miR393* in response to nitrate (Vidal et al., 2010). All four mRNAs (*TIR1*, *AFB1*, *AFB2* and *AFB3*) are cleaved by *miR393* derived predominantly from *miR393b* in developing leaves (Ammour et al., 2011). In rice there are two members of the *miR393* family named *miR393* and *miR393b*. But only *miR393* not *miR393b* is regulated by salinity and alkaline stress (Gao et al., 2010).

Therefore, according to the results it is evident that *TIR1* transcripts may not be subjected to *miR393* cleavage in response to salinity, osmotic stress and ABA. Also the *AFB3* transcripts are not likely to be degraded by *miR393* in response to ABA; however, *miR393* may degrade *AFB3* transcripts in response to salinity and osmotic stress. It is also possible that the down-regulation of *AFB1* and *AFB2* in response to salinity, osmotic stress and ABA is due to the cleavage of their transcripts by *miR393*. Interestingly, even though *miR393a* and *miR393b* are induced by gibberellic acid, it also induces all the auxin receptor genes. This induction may serve to maintain adequate level of transcripts of auxin receptor genes, or alternatively, *miR393* may have a different function in response to GA. Depending on the level of induction in expression, *miR393* may be mainly derived from *miR393b* with respect to salinity, osmotic stress and ABA.

However, this possibility needs to be further investigated. The newly identified siRNAs derived from *AFB2* and *AFB3* (Ammour et al., 2011) are additional components that have to be considered as regulating the level of auxin receptor transcripts in response to salinity, osmotic stress, ABA and GA, as *AFB2* and *AFB3* expression is regulated by above conditions.

Auxin receptor mutants show different levels of resistance to salinity, osmotic stress, ABA and GA

In order to support the histochemical and molecular genetic data, a phenotypic study was carried out using auxin receptor mutants. The seeds were grown on media containing concentrations of NaCl, mannitol, ABA or GA at which more than 50% of wild type seeds were arrested after germination. The percentage of seedlings with green cotyledons was calculated as an indicator of response to the above treatments. Single, double, triple and quadruple mutants were used in this study. However, as mutants were not available in the same ecotype, mutants generated in two different ecotypes (Ws and Col-0) were used. Although it is difficult to draw definitive conclusions since the two ecotypes behave differently in response to same stress conditions, some important observations can be used to explain the involvement of *TIR1/AFBs* in stress response. All four receptor mutants are resistant to NaCl and mannitol, suggesting their common function in responding to the above stresses. Even though all are resistant, they confer different levels of resistance. The discrepant resistance indicates the differential contribution of each receptor in auxin signaling in response to salinity. It is also possible that the severity of the mutant, depending on the site of the mutation in each receptor

gene, confers different levels of resistance to above conditions. *TIR1* makes the major contribution followed by *AFB2*, *AFB3* and *AFB1*, respectively. The double mutant *afb2/afb3* and triple mutant *afb1/afb2/afb3* show very high resistance, suggesting their synergistic effect. However, the *afb1/afb3* double mutant shows a low level of resistance probably due to the stronger effects of *TIR1* and *AFB2*, which make the greatest contribution to the auxin signaling (Parry et al., 2009). As *TIR1* expression is induced by above conditions it may modulate transcription of genes via SCF^{TIR1} required for salinity and osmotic stress responses. Therefore, the misregulation of target gene expression by auxin receptor mutants may result in their resistance to salinity and osmotic stress. The distantly related receptor homolog mutant *afb5-1* also shows moderate resistance to osmotic stress but not to salinity stress. Interestingly *tir1-9* in *Ws* ecotype shows resistance to NaCl and mannitol while *tir1-1* in *Col-0* ecotype shows sensitivity to the same conditions. Therefore, it is likely that there are other genetic factors causing above differences that have to be further investigated. It is also important to notice that *tir1-9* is a knockout mutant but *tir1-1* has a point mutation. Therefore the absence of TIR1 in *tir1-9* and presence of defective *TIR1* in *tir1-1* may also be a reason for the above observation.

The single mutant *afb2-1* shows the highest resistance to ABA followed by *tir1-9*, *afb3-1*, *afb1-1* and *afb5-1*, respectively. Therefore, *AFB2* may have the most important role in auxin-ABA crosstalk. Similar to their resistance to salinity and osmotic stress, double mutants are also resistant to ABA. However, the *afb1-1/afb2-1/afb3-1* triple mutant does not show a synergistic effect; rather it exhibits an epistatic effect (Figure 8.4 b) by *afb1-1* over *afb2-1* and *afb3-1* because, *afb1-1/afb2-1/afb3-1* triple mutant does not

exceed the level of resistance of *afb1-1*. Interestingly, in contrast to ABA resistance, all the auxin receptors except *afb1* show completely opposite response to gibberellic acid. While *tir1-9*, *afb2-1*, *afb3-1* and *afb5-1* are sensitive to GA, *afb1-1* shows resistance. Therefore, *AFB1* might function as a negative regulator of auxin signaling, opposite to other receptor genes in auxin-gibberellic acid crosstalk. The *afb1-1/afb3-1* double mutant and *afb1-1/afb2-1/afb3-1* triple mutant also show resistance, suggesting the strong effect of *afb1-1* over the others. Surprisingly, the *afb2-1/afb3-1* double mutant shows resistance to GA, which cannot be explained without further investigations.

If a phenotype shown by a mutant is its own direct effect, over expression of the wild type gene in mutant background should generally recover the phenotype. Therefore, the sensitive phenotype shown by *tir1-1* in Col-0 ecotype in response to salinity, osmotic stress, ABA and GA was complemented with *TIR1* over-expression in *tir1-1* background. Interestingly, *TIR1* over-expression recovered the sensitive phenotype of *tir1-1* in response to salinity, osmotic stress and GA, and partially recovered the sensitivity to ABA (Figure 9.1). These results provide strong evidence that *TIR1* is directly involved in modulating auxin signaling in response to salinity, osmotic stress, ABA and GA. A transgenic line over-expressing *AFB1* in *tir1-1*[Col-0] was used to understand the *AFB1* function in stress responses as it showed some differences compared to other auxin receptor proteins. Previous work has shown that although both *TIR1* and *AFB1* interact with auxin, neither *AFB1* nor *AFB2* complement the auxin resistant root phenotype of *tir1-1* (Parry et al., 2009). According to the germination assay, *tir1-1* and wild type Col-0 were sensitive to both NaCl and mannitol, and over-expression of *AFB1* resulted in resistance to these stresses. However, results show that *tir1-1* phenotype in response to

ABA and GA can be complemented by over-expression of AFB1 in a *tir1-1* background, suggesting that both TIR1 and AFB1 may have redundant function in response to these hormones.

The *TIR1* promoter contains a *bona fide* ABA responsive element (ABRE)

The interaction of ABRE with transcriptional factors ABFs/AREBs induces the ABA responsive genes (Raghavendra et al., 2010). Therefore, the majority of ABA induced genes contain the consensus ABRE sequence ACGTGG/TC. The core motif ACGT is the binding site for ABFs (Choi et al., 1999). In a study to determine ABRE sequence requirements for ABA induction, it was found that the change in G, T or C following ACGT core motif abolishes the induction by ABA (Hattori et al., 2002).

The *TIR1* promoter contains a putative ABRE, 141 bp upstream of the putative start codon. *TIR1* expression is induced by ABA as discussed above. In order to determine the validity of the putative ABRE in *TIR1* promoter, ACGTGTC was changed to ACGTCTC to generate *TIR1*_{mABRE}::*GUS* (mutated) construct. As expected, ABA does not induce *TIR1*_{mABRE}::*GUS* expression (Figure 12), suggesting that this is a *bona fide* ABRE sequence. Therefore, the induction of *TIR1* in response to ABA may be mediated through the binding of ABFs to ABRE.

Bioinformatics analysis of F-box auxin receptor promoters

In an effort to analyze the auxin receptor promoters using ATHENA, ATHAMAP, PROMOTER web base applications and AGRIS data base, several *cis*-regulatory elements related to salinity, osmotic stress, ABA and GA were identified.

Among them, ABRE like, At MYC2 BS RD22, At MYB2 BS RD22, GARE and drought response element were the most prominent regulatory sequences found. The ABRE like *cis*-regulatory element present in *TIR1* promoter was confirmed as a *bona fide* ABRE as discussed above. *AFB1* also contains an ABRE like sequence. However, it has a higher *e*-value compared to the ABRE in *TIR1* promoter suggesting a lower possibility of being a genuine ABRE. The other two receptor genes *AFB2* and *AFB3* do not contain recognizable ABREs in their promoters. A drought response element, which is a target site for DREB (dehydration responsive element binding) factors (Agarwal et al., 2006), is located in *TIR1* promoter. MYC2 and MYB2 are two transcription factors that bind to ABA inducible gene *RD22*. It is also known that some of the other ABA inducible genes contain MYC2 and MYB2 binding sequences similar to those found in *RD22* (Abe et al., 2003), which are called At MYC2 BS RD22 and At MYB2 BS RD22. Interestingly, all the F-box auxin receptor genes contain MYC2 BS RD22 elements. MYB2 BS RD22 is also present in all genes except *TIR1*. Gibberellic acid responsive elements are responsible for the induction of many GA responsive genes. All the auxin receptor genes are induced by GA. The presence of GAREs in all the receptor genes suggests the involvement of GA in their induction. Nevertheless, validity of these putative *cis*-regulatory elements has to be experimentally determined. However, the induction of *TIR1* in response to osmotic stress and ABA can be explained by the presence of ABA response element and drought response element in its promoter. Also the *AFB3* induction by ABA might be mediated through MYC2 BS RD22, MYB2 BS RD22 or both. Nevertheless, down-regulation of *AFB1* and *AFB2* in response to ABA despite the presence of relevant *cis*-regulatory elements has to be addressed.

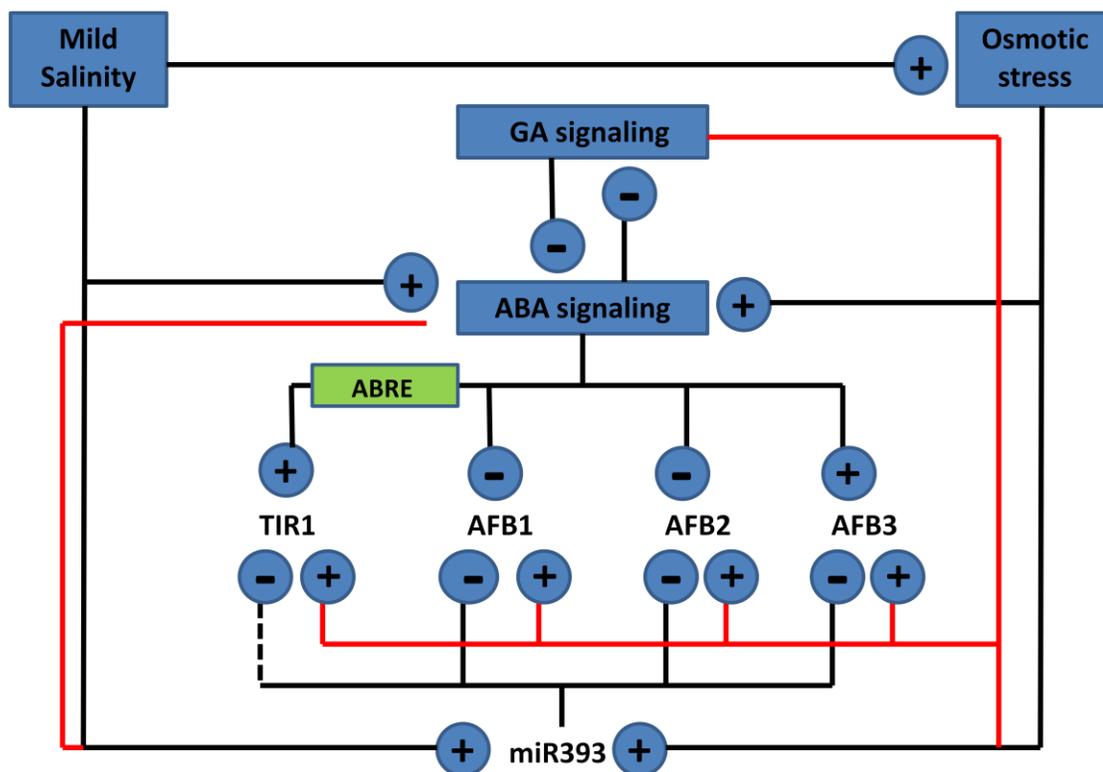


Figure 13. A schematic diagram to illustrate possible regulation of *TIR1*/*AFBs* in response to salinity, osmotic stress, ABA and GA. “+” indicates the up-regulation and “-” indicates the down-regulation. Salinity and osmotic stress enhance ABA signaling. GA and ABA act antagonistically. Salinity and osmotic stress up-regulate *TIR1* but down-regulate *AFBs*. ABA up-regulates *TIR1* and *AFB3* but down-regulate *AFB1* and *AFB2*. Up-regulation of *TIR1* by ABA may occur through ABRE. GA up-regulates all four receptor genes. Salinity, osmotic stress, ABA and GA up-regulate *miR393*. *miR393* may down-regulate *AFBs* but not *TIR1* in response to salinity and osmotic stress. It may down-regulate *AFB1* and *AFB2* but not *TIR1* and *AFB3* in response to ABA. *miR393* may not down-regulate auxin receptor gene expression in response to GA.

Conclusions and future directions

Auxin receptor *TIR1* is up-regulated by mild salinity and osmotic stress while *AFB1*, *AFB2* and *AFB3* are down-regulated. Highly induced *miR393* in response to above conditions may be responsible for the down-regulation of *AFBs*. However, *TIR1* transcripts are less likely to be degraded by *miR393* in response to mild salinity and osmotic stress. Different levels of resistance shown by auxin receptor mutants suggest their degree of influence to adapt the plant to above conditions. According to this analysis, *TIR1* makes the major contribution to stress response, followed by *AFB2*, *AFB3* and *AFB1* respectively. ABA up-regulates *TIR1* and *AFB3* but down-regulates *AFB1* and *AFB2*.

ABA responsive element binding factors may bind to the ABRE found in *TIR1* promoter and enhance the transcription. *miR393* may degrade *AFB1* and *AFB2* transcripts in response to ABA. *AFB2* may play the major role in auxin-ABA crosstalk according to the physiological data. Gibberellic acid induces all receptor genes as well as *miR393*. *AFB1* may act as a negative regulator of auxin signaling in response to GA while others act as positive regulators.

There are many avenues of this project to be further studied. It is important to understand how *miR393* targets specific receptor genes under different environmental conditions. Also the decisive factor of the *miR393* origin in response to above conditions has to be investigated. It is also interesting to know which genes are transcribed via SCF^{TIR1} and SCF^{AFBs} in response to salinity, osmotic stress, ABA and GA. All the putative regulatory elements found in auxin receptor genes may not be functional. Therefore, experimental analysis of most probable regulatory elements will give insight

into the gene expression data. The GARE found in *TIR1* was experimentally analyzed by site directed mutagenesis in this study. Therefore, better understanding of the underlying molecular mechanism of SCF^{TIR1/AFBs} mediated auxin signaling in response to different hormones and environmental cues will complete the gaps in plant hormone signaling networks.

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