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Characterization of SABP2-Interacting Proteins (SIP) 428: an NAD+-Dependent Deacetylase Enzyme in Plant Abiotic Stress Signaling

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by

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ABSTRACT

Characterization of SABP2-Interacting Proteins (SIP) 428: an NAD^+-Dependent Deacetylase Enzyme in Plant Abiotic Stress Signaling

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Abiotic stress leads to a change in the water content of plants. Salinity and osmotic stress affect both the morphology and physiology of plants. Plants have therefore responded to these environmental changes by adapting and tolerating them. The SABP2-interacting proteins (SIP) 428-silenced RNAi transgenic tobacco lines were subjected to various abiotic stresses (salinity, osmotic, and drought). The effect of SIP428-silencing on the tobacco plants subjected to these abiotic stresses was monitored. The results from the root growth data show that the sip428-silenced lines exhibit enhanced tolerance to the stressors compared to the wild-type plants. Interestingly, results of the relative chlorophyll content show no significant difference between the wild-type plants and sip428-silenced transgenic plants. In summary, based on the results presented in this study it could be concluded that SIP428 is a negative regulator of salinity, osmotic and drought stresses. Further studies are required to understand the mechanism.

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CHAPTER 1. INTRODUCTION

Plants survive stresses by modifying their metabolism. Plants differ from humans in that when pathogens or abiotic stresses pose a threat, they can activate the initiation of several defense mechanisms that inhibit the growth and spread of pathogens thus conferring stress tolerance. The occurrence of extreme environmental conditions of cold, high temperature, light, drought leading to detrimental internal factors with consequences on nutritional or hormonal imbalance adversely affect plants causing them to initiate and utilize these stress tolerance mechanisms and signaling pathways. The communication of signal occurs when it is received by receptors in the cytosol, cell membrane or it is attached to a signaling molecule so that a response is produced and a conformational change of receptor occurs. As additional measures, physical barriers in their cell wall, which provides a site where chemical defense molecules such as lignin, suberin, and callose can be deposited can occur at the cellular sites of infection to prevent the entry of pathogens are used as protection (Luna et al. 2011; Malinovsky et al. 2014).

In general, there are two types of resistance in plants: local and systemic acquired resistance. The local resistance is initiated when the plant is able to recognize the existence of unusual events or through molecular patterns associated with a pathogen which can be found on the cell at the site of infection (Zipfel 2008). For this recognition to occur, the resistance proteins activate a hypersensitive response which activates enzymes, reactive oxygen species, defense responses, and cell death (Guidetti-Gonzalez et al. 2007). The systemic acquired resistance activates a response in distal tissues, helping the plants recovery either from disease or stress. For systemic acquired resistance to occur, the presence of endogenous molecules such as jasmonate, salicylate, and/or pathogenesis-related proteins are required.

The goal of this research is to characterize and understand the role of SIP428 in plant defense mechanisms.

Plant Immune System

Changing environmental conditions and pathogen attacks have made it necessary for plants to develop a complicated immune system. Based on modification and type of host, there are generally two main types of innate immunity. The first is pathogen-triggered immunity (PTI) and the second is effector-triggered immunity (ETI), in which the plants recognize specific molecular patterns and pathogen-derived effectors to start a defense response.

Host-pathogen refers to a pathogen that can evade all the physical barriers of a plant and lead to disease. Plants exhibit both host-pathogen and gene-for-gene resistance. There is the possibility that salicylic acid-mediated signaling, the expression of pathogenesis-related genes, and other defense genes are involved in this resistance (Kumar 2014). In plants, the host-specific defense is due to the induction of cognate resistance proteins in response to microbial avirulent protein. The absence of this response leads to the onset of disease (Dangl and Jones 2001). With respect to non-host resistance, the majority of plant species exhibit resistance to most pathogens. Non-host pathogens do not have the ability to escape the host defense mechanisms and therefore, cannot cause disease. Non-host resistance occurs due to the inability of the pathogen to adapt to the physiology and growth pattern of the plant. The ability of the plant to recognize either the invading pathogen or products of the pathogen and subsequent activation of defense responses leads to hypersensitive response-related apoptosis (Kamoun et al. 1999; Thordal-Christensen 2003;Senthil-Kumar & Mysore 2013). Pathogenesis-induced hypersensitive response mitigates the penetration and spread of pathogens through cell death localized at the infection site. Salicylic acid and other metabolites play a significant role in the regulation of cell death and hypersensitive response.

Plant Stress

From environmental to biotic stress, plants encounter numerous harsh conditions. In plants, abiotic stresses alter plant growth, metabolism, and crop production. For plants to survive, their metabolic cascade undergoes modifications through a response mechanism and recovery after the stress has ceased (dos Reis et al. 2012; Ghosh & Xu 2014). Modifications such as hardening of the cell wall, reducing root length, and alteration in membrane characteristics are caused by abiotic stress-responsive pathways (Atkinson and Urwin 2012; Petricka et al. 2012). This stress causes changes both biochemically and physiologically. For instance, stress such as chilling which occurs below 0^{o}C affects the structure of the cell, metabolic function and causes solute leakage; drought stress results in stomatal closure, loss of water which affects growth rate, assimilation of carbon, damages the membrane and causes the unusual activity of CO_2 fixing enzymes that should help decrease reactive oxygen species generation (Farooq et al. 2009; Abogadallah 2010).

As industrialization increases, so does the generation of toxic metals such as nickel and zinc, that subsequently causes toxicity in plant tissues. Salinity stress, implicated to be one of the most devastating abiotic stresses, has a negative effect on the biochemistry and physiology of plants. High salt content elevates the level of ion toxicity and reactive oxygen species, affects nutrient deficiency, cell turgidity, and reduces the water content of the leaf (Munns & Tester 2008; Khan et al. 2014). Drought stress affects plant development and growth with effects on physiological processes leading to changes in biochemistry, reactive oxygen species (ROS) accumulation, toxins, and formation of protective secondary metabolites such as terpenoids and phenolics (Yadav et al. 2021). Waterlogging causes flooding stress which gives rise to anoxia causing a shortage of oxygen in submerged plant parts. Ozone stress elevates the level of reactive oxygen species generated. The temperature on a global scale has been unstable due to climate

change and this has a damaging effect on plants (Hasanuzzaman et al. 2013). Temperature stress affects the physiology, biochemistry, and metabolism (depending on the length of exposure) of plants leading to the activation of molecular mechanisms and expression of genes which helps to induce response(Kazemi-Shahandashti et al. 2014; Siboza et al. 2014). Plants' responses to these stresses are usually by expressing certain genes which in turn leads to the process of detoxification, restoring homeostasis and growth (Xiong and Zhu 2002).

Signaling Pathways in Plant Immunity

There are several signaling pathways with a role in plant immunity. Jasmonic acid (JA), one of the signaling molecules in plant defense, activates protective defense to mechanical injury of plant tissues and to plant attacks; an increase in JA concentration leads to ubiquitin-mediated proteolysis of repressor transcription factors which eliminates the suppression of jasmonic acid gene expression (Kepinski 2007). Salicylic acid (SA), another signaling molecule in plant defense, is a beta hydroxy phenolic metabolite and plant hormone that has a crucial function in many aspects of plants life including defense against biotic and abiotic stresses (Vlot et al. 2009). SA plays a major role in plant innate immunity and SAR, regulation of stomatal closure, and seed germination(Lee et al. 2010; Prodhan et al. 2018). The SA-mediated signaling pathway regulates a myriad of responses ranging from immunity to pathogens, leaf senescence, respiration, drought tolerance, stomatal closure, plant development, flowering, etc. (Munné-Bosch & Peñuelas 2003; Martínez et al. 2004; Norman et al. 2004; Corina Vlot et al. 2009; Lee et al. 2010; Dempsey & Klessig 2017; Prodhan et al. 2018).

Salicylic Acid Biosynthetic Pathway

SA is a chemical messenger regulating biological processes at low concentrations (An and Mou 2011; Fu and Dong 2013). SA can be generated via two enzymatic pathways that

require the primary metabolite called chorismate, the product of the shikimate pathway (Garcion et al. 2008). The two pathways (Fig. 1), phenylalanine ammonia-lyase (PAL) and isochorismate synthase (ICS) involves the conversion of chorismate into salicylic acid through a series of enzymatic reaction using benzoate intermediates or coumaric acid (Wildermuth et al. 2002; Strawn et al., 2007). SA can be converted into various derivatives including transportable (methyl salicylate, MeSA) and storage forms such as glucosylated SA derivatives which are synthesized in the cytoplasm but stored in the vacuole (Dean et al. 2005). Positive and negative regulation of SA-mediated biosynthesis is essential (Ding and Ding 2020). The three key genes that define the isochorismate pathway (ICS1, Enhanced Disease Susceptibility 5(EDS5), PBS3) encode enzymes that define the pathway and play role in SA biosynthesis and metabolism along with the transport of metabolites such as salicylic acid glucoside (SAG) within organelles (Nobuta et al. 2007; Garcion et al. 2008; Rekhter et al. 2019). Although SA is crucial for plant cell function, its constitutive accumulation because of the synthesis from both *de novo* biosynthesis and metabolic release from inactive forms is detrimental to plant fitness (Manthe et al. 1992; Šašek et al. 2014; Janda & Ruelland 2015). This is accounted for by the tight regulation of components of the pathways by the expression of transcriptional regulators. These regulators control the accumulation of biotic-stress-induced SA: Genes such as *EDS1*, *Phytoalexin Deficient 4* (*PAD4*), and *Senescence Associated Gene 101* (*SAG101*) (Vlot et al. 2009). The two pathways are evolutionarily conserved across species and mutations in these pathways in plants led to an increase in susceptibility to many pathogens suggesting that they are required for SA function in stress responses (Vlot et al. 2009).

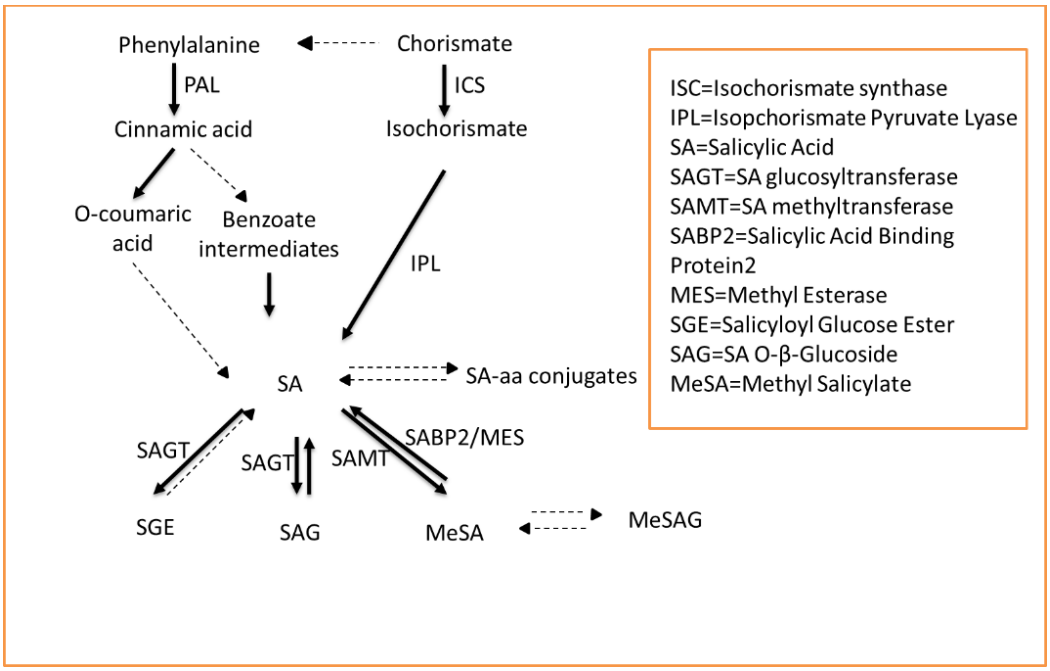

Figure 1. **Salicylic acid signaling pathway (**Redrawn from (Vlot et al. 2009)

Salicylic Acid-Binding Proteins

Salicylic acid (SA) is a phenolic compound synthesized by plants with implications in seed germination, growth, senescence, response to abiotic stress and stomatal closure (Morris et al. 2000; Rajjou et al. 2006; Stacey et al. 2006). The effects of salicylic acid can also be indirect as SA inhibits biosynthesis or signaling of other phytohormones such as jasmonic acid (Vlot et al. 2009)

Salicylic acid-binding protein 2 (SABP2) from tobacco leaves exhibits a high affinity for SA and is an important component in the salicylic acid signaling pathway (Du & Klessig 1997). This protein offers a paradigm for hormonal regulation in plants. SABPs are essential for primary metabolism, eliminating these results in an undefined anomaly in plant growth. Kumar and Klessig reported that when SABP2 was silenced using the RNA interference technique, the

result was a reduction in resistance to *tobacco mosaic virus* and SA-induced pathogenesis-related gene expression (Kumar & Klessig 2003).

SABP2 is a 29kDa esterase-like enzyme that catalyzes the conversion of the signal molecule methyl salicylate (MeSA), into salicylic acid (SA) which has a significant role in the signal transduction pathway in plants (Kumar and Klessig 2003; Forouhar et al. 2005; Park et al. 2007; Kumar & Klessig 2008). SA, the product of the reaction, binds in the active site of SABP2 thereby inhibiting its enzymatic activity. SABP2 has been established as a key enzyme that likely helps in fine-tuning the cellular levels of SA and induction of MeSA mediated SAR in distal tissues (Forouhar et al. 2005; Park et al. 2007; Kumar and Klessig 2008). SABP2 has been shown to be involved in SA-mediated SAR signaling in tobacco, potato, *Arabidopsis,* and other plants. Reports indicate that SABP2 interacts with cellular proteins to initiate downstream signaling and activate responses leading to resistance (Kumar et al. unpublished). Several SABP2-interacting proteins (SIP), including SIP428, have been identified using a yeast-two-hybrid screening (Haq et al. 2020).

The Silent Information Regulator 2 Family

The SIP428 was identified in a yeast two-hybrid screen and subsequently verified using a glutathione s-transferase (GST) pull-down assay (Haq et al. 2020). SIP428 has been shown to belong to the silent information regulator (SIR) 2 family using genetic sequencing and bioinformatics analysis. They are a family of (nicotinamide adenine dinucleotide) NAD+ dependent protein deacetylases called sirtuins which were grouped with the yeast SIR2. They have been shown to have a significant role in the regulation of biological pathways.

This group of proteins can be found in diverse organisms ranging from bacteria, mammals, and even plants. Two sirtuins are encoded in bacteria and archaea while there are

seven in humans (SIRT 1-7), yeasts have five, and *Arabidopsis thaliana* and *Oryza sativa* both have two (SRT1 and SRT2) (Frye 2000). The sirtuins occupy different subcellular compartments ranging from the nucleus to the mitochondria (Blander and Guarente 2004; North and Verdin 2004; Michishita et al. 2005; Wen et al. 2005; Haigis et al. 2006; Tanno et al. 2007; König et al. 2014). Their dependence on NAD^+ suggests that their enzymatic activity has a direct link to cell's energy level through NAD^+/NADH or nicotinamide (NAM) ratio (Lin et al. 2000; Lin et al. 2002; Bitterman et al. 2002; Anderson et al. 2003; Lin et al. 2004).

Lysine acetylation is a reversible post-translational modification playing role in the regulation of transcription (Xiong et al. 2016), activation, and deactivation of certain pathways. It neutralizes the positive charge of the amino group when the acetyl group is transferred to lysine, affecting protein function such as the activity of enzyme activities, the interaction between proteins, and DNA (Yang & Seto 2008). For the cell signaling by lysine acetylation, three classes of proteins—lysine acetyltransferase (KAT), lysine deacetylase (KDAC), and proteins recognizing and binding acetyl-lysine. Acetyl-CoA is a lysine acetylation substrate in enzymatic reactions catalyzed by lysine acetyltransferases (KATs) (Yuan & Marmostein 2013; Drazic et al. 2016). Non-enzymatic lysine acetylation can occur at pH higher than 8 and high Acetyl-CoA concentrations; both conditions are present during active respiration in the mitochondrial matrix and chloroplast stroma during photosynthesis (Wagner and Payne 2013; König et al. 2014; Baeza et al. 2015; Hosp et al. 2017).

The SIR2 family is characterized by acetylation of lysine residues in the tail of their histones (Braunstein et al. 1993) belonging to the class III histone deacetylases which have been implicated in increased replicative life span (Guarente 1999; Baur et al. 2006; ; Houtkooper et al. 2012; Sebastián et al. 2012), activation of survival mechanisms such as senescence, inhibition of

apoptosis and activation of stress response pathways (Cohen et al. 2004). Recently, they have been shown to not only deacetylate histone proteins but that they also deacetylate non-histone proteins (Yang & Seto 2008)

In terms of preferences and targets for the deacetylation of different plant proteins, very little is known. Studies on *Arabidopsis* sirtuins show that there is lysine-acetylation of tricarboxylic acid (TCA) cycle enzymes such as isocitrate dehydrogenase (Wang et al. 2010; Zhao et al. 2011), and deacetylation of limited mitochondrial protein such as the adenosine triphosphate (ATP/ADP) carrier (König et al. 2014). It has been demonstrated that, for human liver cells and bacteria, lysine acetylation depends on the nutrient status of the cell, and acetylation of metabolic enzymes regulates the activity of glycolysis and TCA cycle (Wang et al. 2010; Zhao et al. 2011). Current knowledge about plant sirtuin is still limited, but there have been several suggestions of their roles in genomic stability, cell oxidative damage (Huang et al. 2007a), gametogenesis, fruit development, and ripening (Zhao et al. 2015).

Several compounds are known to inhibit or activate SIR2 activity. Splitomicin, an inhibitor of NAD+ dependent deacetylase activity of SIR2, disrupts silencing at HML, HMR, and telomeric loci in budding yeast (Bedalov et al. 2001). Sirinitol, another inhibitor of SIR2, interferes with the formation of the body axis in *Arabidopsis thaliana* (Grozinger et al. 2001). After deacetylation by SIR2, an NAD+ molecule is cleaved to produce nicotinamide and O-acetyl-ADP-ribose (Fig. 2) (Tanner et al. 2000; Tanny & Moazed 2001). This product, nicotinamide, works as an inhibitor of SIR2 activity *invitro* and *invivo*. This was tested by applying nicotinamide to yeast cells (Bitterman et al. 2002), and it was observed that it derepresses target loci, increases recombination of recombinant DNA loci, and shortens life span. Apicidin and Trichostatin A have also been identified as deacetylase inhibitors(Darkin-

Rattray et al. 1996). Polyphenol compounds such as quercetin, resveratrol, and piceatannol activate deacetylase activities, leading to an increase in life span after DNA damage at low concentration (Howitz et al. 2003).

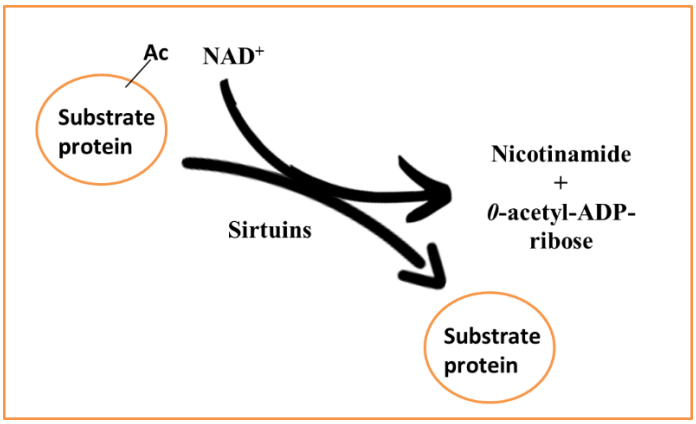

Figure 2. **Sirtuin deacetylation reaction.** NAD^+-dependent deacetylation catalyzed by sirtuins to produce a deacetylated protein, nicotinamide, and 2-O-acetyl-ADP ribose (Tanner et al. 2000).

Localization of SIR2

Sirtuins are ubiquitously expressed with a conserved catalytic core of 275 amino acids (Brachmann et al. 1995). Their diversity in mammals is due to their specialized function and cellular localization. The first known sirtuin found in yeast functions in the regulation of chromatin structure and gene expression is localized in the nucleus, this is the case for SIRT1, 6, and 7 (Michishita et al. 2005). SIRT2 is localized in the cytoplasm and functions in deacetylating transcriptional regulators and influencing chromatin compaction (Jing et al. 2007; Vaquero et al. 2006). SIRT3,4 and 5 are localized in the mitochondria (Haigis et al. 2006; Schlicker et al. 2008). It has also been reported that some of these proteins shuttle between compartments in response to different stimuli (Tanno et al. 2007). SIP428 has been reported to

be a non-histone protein and subcellular localization has shown it to be localized in the mitochondria (Thakuri 2018). SIP428 has shown high homology to human sirtuin 4 (SIRT4) based on bioinformatic analysis (Thakuri 2018). Mitochondria are central mediators and cellular powerhouses of metabolism and production of energy, with a plethora of molecular building blocks and adenosine triphosphate (ATP) needed for development and growth (Wallace 2005; Hosp et al. 2017). The ability to oxidize amino acids, fatty acids, and sugars, by reduction of molecular oxygen and creation of proton gradient across inner mitochondrial membrane couples' mitochondria to ATP production (Baeza et al. 2015). Mitochondria have been implicated in many fundamental cellular processes, some of which has links with providing precursors for anabolic processes, coordination of nucleus-mitochondrion communication and acting as guard for cellular health (Baeza et al. 2015). A high degree of regulation of mitochondria protein complement is required as mitochondria works in tandem with subcellular metabolic pathways and organelles (Hosp et al. 2017). Mitochondria have been said to undergo lysine acetylation which is a major regulatory mechanism in modulation of protein function (Baeza et al. 2015).

Role of SIR2 In-Plant Defense

Sirtuins appear to have an affinity for sensing changes in the environment. Sirtuins are present in plant species such as *Arabidopsis thaliana*, *Oryza sativa* (Huang et al. 2007; Zhong et al. 2013). Functional studies of plant sirtuins so far indicate that they are of importance in the regulation of plant growth and their responses to biotic and abiotic stresses are being investigated. Acylation/deacylation of histone proteins has been shown to serve as an epigenetic switch with a role in regulating cellular processes through the modulation of gene expression since histones coexist with DNA in the nucleosome (Zhao et al. 2018). The deacetylation of H3K9Ac by AtSRT1 in *Arabidopsis thaliana* led to repression of stress-responsive genes such as

ZAT10, LOS2, RD29A, and *RD29B,* at their promoter regions (Liu et al. 2017b). It exhibits suppressive action towards the glycolytic pathway by repressing gene expression and function of certain enzymes such as hexokinase, pyruvate kinase, phosphofructose kinase, enolase in response to abscisic acid (ABA), NaCl, and mannitol treatments, all of which are abiotic stresses (Liu et al. 2017). The AtSRT2B localized in the nucleus uses histone as its native substrate and has been shown to exhibit suppressive action against the local response of *Arabidopsis* to *Pseudomonas syringae* pv. tomato DC3000, a bacterial pathogen implicated in biotic stress and toward expression of SA biosynthetic genes *PAD4, EDS5, SID2* (Wang et al. 2010). In rice, OsSRT1 localized in the nucleus was shown to deacetylate H3K9Ac of genes with function in a variety of metabolic pathways or in stress response as well as H3K9Ac deacetylation on DNA transposons and retrotransposons (Zhang et al. 2017; Zhong et al. 2013). RNAi-mediated silencing of OsSRT1 increased H3K9Ac levels, hydrogen peroxide production, fragmentation of genomic DNA, and apoptosis while overexpression showed that there was an increase in tolerance to paraquat challenge indicating its importance in safeguarding rice against genomic stability, cell damage, and oxidative stress (Huang et al. 2007). The expression of *SIP428* transcripts was shown to be downregulated upon TMV infection (Haq et al. 2020). Also, transgenic tobacco plants lacking *SIP428* (via RNAi silencing) exhibited enhanced basal resistance and stronger activation of SAR (Thakuri 2018). The studies using the SIP428-silenced plants also showed that SIP428 acts upstream of SABP2 in the SA-signaling pathway (Thakuri 2018).

Previous Studies on SIP428

The role of SIP428 in basal and systemic acquired resistance was investigated using *Pseudomonas syringae* pv *tabaci* (*Pst*), a virulent tobacco pathogen, and Tobacco Mosaic Virus

(TMV)(Haq et al. 2020). Results showed that sip428-silenced plants allowed a significantly lower *Pst* growth as compared to wild-type plants. These results indicated that SIP428 negatively regulates basal resistance and systemic acquired resistance (Thakuri 2018). Expression of *SIP428* transcripts was mildly downregulated 48 hours post-TMV infection (Haq et al. 2020). IT was concluded that SIP428 is a deacetylase but not a histone deacetylase (Thakuri 2018).

Hypothesis

SIP428 has a crucial role in conferring immunity to plants. Reports from other studies have shown that sirtuins in plants play a role in plant stress. Therefore, it is hypothesized that SIP428 confers tolerance to abiotic stress. Hence, after treatment of sip428-silenced lines with stress-inducing chemicals such as sodium chloride (salinity stress), mannitol (osmotic stress), and polyethylene glycol (drought stress) it is expected that these plants will be susceptible and have a reduced rate of photosynthesis. To test the role of SIP428 in abiotic stress, the sip428-silenced tobacco lines were treated with various abiotic stress-inducing chemicals such as sodium chloride (salinity stress), mannitol (osmotic stress), and polyethylene glycol (drought stress). The effect of abiotic stress on the plants was recorded and analyzed.

CHAPTER 2. MATERIALS AND METHODS

Plant Materials

Tobacco plants, wild-type *Nicotiana tabacum cv. Xanthi-nc NN* (XNN), and sip428-silenced in the *N. tabacum cv. Xanthi-nc* NN background (transgenic lines #MS1-2, MS1-2-7, and MS1-2-14) were used in this study (Thakuri 2018). The tobacco seeds were sown on autoclaved soil (Berger BM1, All-purpose mix) in 4 x 4-inch square plastic pots in a plant growth chamber set at 22°C and a 16-hour light cycle maintained with a light intensity of about 200 μmol m^{-2} sec^{-1}. Ten days after sowing, individual seedlings with two cotyledons were transferred into 4 x 4-inch pots and grown for approximately three weeks (as stated above). Each young plant was then transferred to a single 7" pot and grown for 2-3 weeks (as stated above) before using them for the experiments. Water-soluble fertilizer (Jack's professional, diluted according to manufacturer's instructions) was added two days after transferring plants to the 7" pot.

Chemicals And Reagents

Sodium chloride (NaCl), methanol, ethanol, acetone, Murashige and Skoog (MS) medium, sucrose, polyethylene glycol 8000 (PEG 8000), and mannitol were from Fisher Scientific; phytoagar was from Phytotech, and Gamborgs vitamins (in-house).

Other Materials and Instruments

For this research, the following instrumentation was used: UV/Vis spectrophotometer (Evolution 300, Thermofisher Scientific), pH meter (Fisher Scientific), microcentrifuge (Eppendorf), micropipettes (Phenix), microcentrifuge tubes (Fisher Scientific), and MultispeQ (PhotosynQ).

Methods

Antibiotic Selection

For the antibiotic selection of the transgenic seeds from sip428-silenced lines, seeds were surface sterilized using 20% bleach (details provided below) and sown on ½ x MS media plates containing 1% sucrose and kanamycin at a final concentration of 100µg/ml. After incubating at 4°C in the dark for 3 days, the plates were placed in a lighted chamber (12 h light) at 25ºC for the seeds to germinate and seedlings to grow. These seedlings were observed for two weeks, and the effect of the antibiotic was visible after two weeks on the wild-type tobacco seedlings with no effect on sip428-silenced seedlings confirming that it is transgenic**.**

Abiotic Stress

Seeds of wild-type *Nicotiana tabacum cv. Xanthi-nc NN* (XNN) and sip428-silenced were incubated in 70% ethanol for five minutes. Then the ethanol was drained out and 20% bleach was added to the seeds. The seeds were incubated in bleach solution for twenty minutes on a rotating shaker. After the bleach was taken out, the seeds were rinsed thrice with autoclaved water and forty seeds each were plated on ½ MS media plates supplemented with 1% sucrose and Gamborgs vitamin (media used in subsequent experiments followed the same protocol)(Gamborg et al. 1968). The plates with seeds were stored at 4°C for 72 hrs to break the dormancy and allow for synchronized germination and later transferred to the controlled environment with 12 hrs of light at 25ºC. Sterile forceps were used to transfer five young seedlings of each line to ½ MS media plates containing different concentrations of salt, mannitol, or PEG. Young seedlings were incubated under 12 h of light in a temperature-controlled room (as above) for 3 weeks. For assessing the effect on root growth, plates with the seedlings were

kept vertical in a rack. After the desired time, plants were photographed, and root length measurements were taken using Image J software as described below.

Salinity Stress

Ten 10-day old seedlings from ½ MS media plates were transferred to ½ MS media plates (as described earlier) supplemented with initial 100mM to 300mM sodium chloride final concentrations. For subsequent experiments, 150mM and 200mM sodium chloride final concentrations were used. Growth was monitored weekly for fourteen days.

Osmotic Stress

Ten 10-day old seedlings from ½ MS media plates were transferred to ½ MS media plates (as described earlier) supplemented with 100mM to 300mM mannitol final concentrations. Then, 150mM and 200mM mannitol final concentrations were used for subsequent experiments. The growth of seedlings was monitored weekly for fourteen days.

Drought Stress

Ten 10-day old seedlings from ½ MS media plates were transferred to ½ MS media plates (as described earlier) supplemented with an initial 1% to 5% final concentrations of polyethylene glycol (PEG)8000. Then, 3% and 5% PEG final concentrations were used for subsequent experiments. Growth was monitored weekly for fourteen days.

Root Length

Ten plants from each of the experimental group were used to determine the root length. For each of the experiments, pictures were taken at 7- and 14-days intervals and uploaded on the

ImageJ software. To analyze the root length, a scale of 1cm was set and the root was traced with the freehand tool. The output was used to calculate the mean and standard error as reported.

Chlorophyll Content

Six-week-old wild-type and SIP428-silenced lines were grown. The plants were watered with solutions of 100mM NaCl, 100mM mannitol, and 3% PEG for 10days. Leaf disks were collected after two weeks using cork borer #6. The samples were weighed, ground to a fine paste using a pre-chilled pestle and mortar using liquid nitrogen, and 80% acetone was added. Chlorophyll a was measured. The chlorophyll a was quantified at a wavelength of 645nm and chlorophyll b at a wavelength of 663nm using a spectrophotometer (Arnon 1949). No significant difference in chlorophyll content was observed. Therefore, only data from chlorophyll a was presented. The relative chlorophyll content was also measured using the "PhotosynQ", a handheld fluorometer/chlorophyll meter directly on leaves attached to the plants as per the manufacturer's instructions. The device was pre-calibrated by placing the panel of the chlorophyll calibration card between the clamps, according to manufacturer's instructions. The relative chlorophyll content is used as a measure of overall health of plant and onset of stress at a wavelength of 650nm and infrared of 940nm (Kuhlgert et al., 2016; Xiong et al., 2015). Six-week-old wild-type and SIP428-silenced lines were grown and subjected to different stresses as described earlier. Absorbance-based data was collected by placing leaves between a MultispeQ connected to a mobile application.

CHAPTER 3. RESULTS AND DISCUSSION

Antibiotic Selection of sip428-silenced Plants

To examine the transgenic seeds from sip428-silenced lines were sown on ½ x MS media plates containing kanamycin 100µg/ml. After incubating at 4°C in a dark room for 3 days, the plates were placed in a lighted chamber (12 h light) for the seeds to germinate and seedlings to grow. These plates were observed for two weeks, and the effect of the antibiotic was visible after two weeks on the wild-type tobacco seedlings with no effect on SIP428-silenced seedlings confirming that it is transgenic**.**

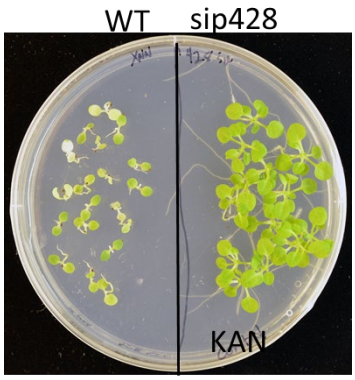

Figure 3. **Antibiotic selection of SIP428-silenced plants** (sip428). Pictures represent two weeks' growth.

Abiotic Stress

To test the hypothesis that SIP428 plays a role in abiotic stress responses, wild type and transgenic plants were treated to a variety of abiotic stress conditions. These included salinity, drought, and osmotic stress. T2 and T3 transgenic lines were tested.

Role of SIP428 in Mediating Salinity Stress

To examine the role of SIP428 in salinity stress, RNAi-mediated MS1-2 sip428-silenced lines were used (Thakuri 2018). In an initial pilot study to test varying concentrations of salt, sip428-silenced seeds from the T2 generation (MS1-2) were used. Ten-day-old seedlings of both the wild-type and the sip428-silenced lines grown in 1/2x MS media were transferred to the MS media plates containing various concentrations (0, 100, 200, and 300mM) of NaCl. The seedlings were allowed to grow under 12 h of light in a temperature-controlled room (set at 22°C). The growth of the seedlings was observed for 14 days. The result shows that the plants were able to grow in 100mM and 200mM NaCl but they failed to grow under 300mM NaCl. This shows that 300mM concentration was too high for both the wild-type and the sip428-silenced tobacco seedlings (Fig. 4). Hence, the seedlings did not grow well. A similar effect was observed when the seedlings were grown to determine the effects of NaCl on the root growth (Fig. 5). Therefore, 150mM and 200mM were used in subsequent experiments.

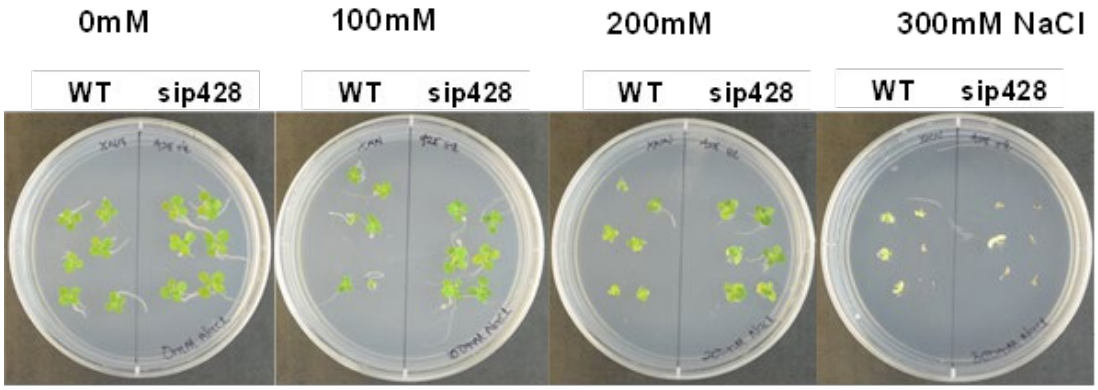

Figure 4. **Growth of MS1-2 sip428-silenced plants under salinity stress**. Seedlings of WT (wild-type) and sip428 lines were grown in 0, 100, 200 and 300mM sodium chloride for 14 days. Experiments were repeated twice with similar results.

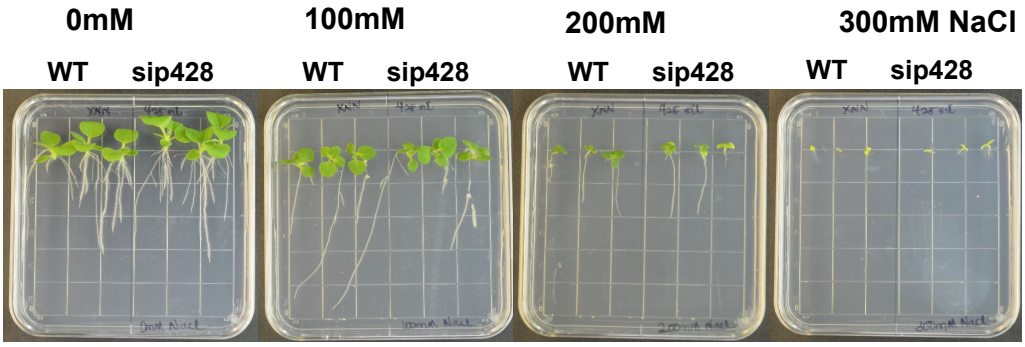

Figure 5. **Growth of roots in MS1-2 sip428-silenced plants under salinity stress**. Seedlings from this experiment were used to determine the root length. Experiments were repeated twice with similar results.

Comparison Between Three SIP428-Silenced Lines Exposed to Salinity Stress

To compare the effect of plant growth in response to intermediate concentrations of stress-inducing chemicals, three different sip428-silenced lines, MS1-2 (T2 generation seeds), MS1-2-7, and MS1-2-14 (T3 generation seeds) were used. Because all three lines are silenced in sip428 expression, it was expected that all three lines would exhibit similar phenotypes upon exposure to sodium chloride. The sip428-silenced lines and the wild-type were grown on MS-media plates containing 0mM, 150mM, and 200mM sodium chloride (Fig. 6, Fig. 7 and Fig. 8) since it had been established that the plants survived better at lower concentrations (Fig. 4). This was done to observe the effect of these varying concentrations of NaCl on the root growth of the seedlings.

MS1-2 (T2 Generation)

The result shows that there is a difference in the root growth in the MS1-2 lines compared to the wild-type tobacco plants. The roots of both the MS1-2 and WT tobacco plants grew to similar lengths when no sodium chloride was added to the growing medium. The difference in the roots of the MS1-2 plants and the wild-type in 150mM and 200mM sodium chloride treated seedlings was statistically significant (Fig. 6 A and B and SD1 in Appendix C). The difference in root growth was more pronounced upon 14 days exposure (6C, and 6D and SD2 in Appendix C) compared to the 7 days (6A and 6B). Contrary to the hypothesis, the knockdown of SIP428 seem to help the MS1-2 sip428-silenced lines thrive better as salt concentration increased, exhibiting a protective effect. It is therefore likely that the sip428-silenced lines can exclude the ion from their tissue or tolerate ion accumulation.

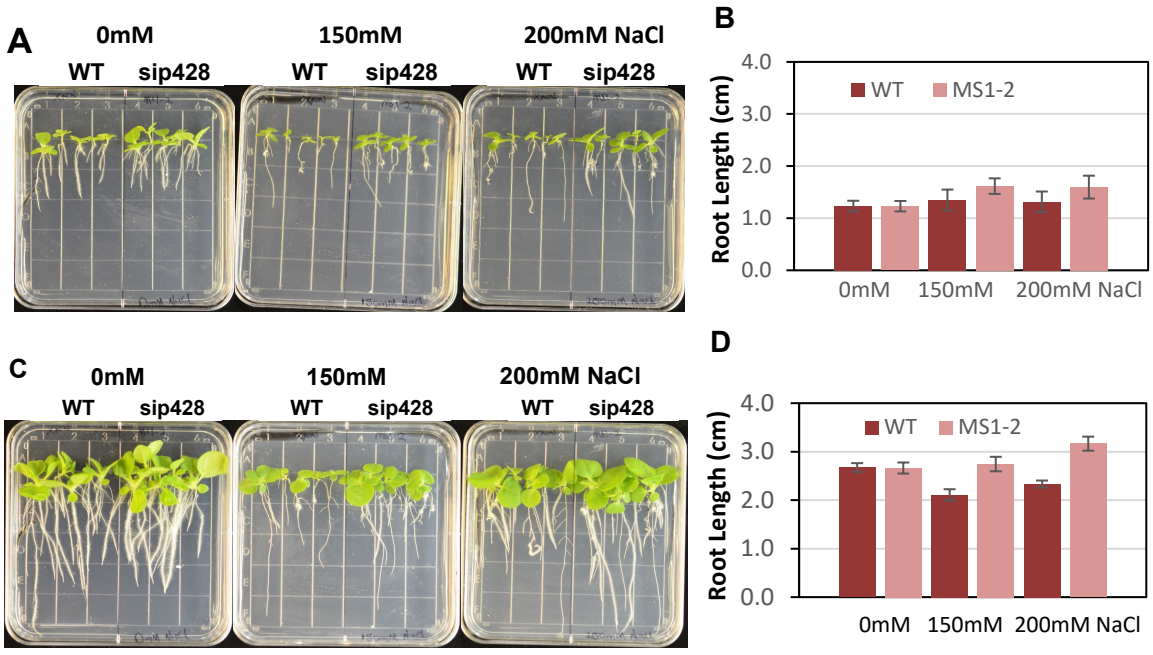

Figure 6. Root Growth of MS1-2 sip428-silenced Plants Under Salinity Stress. A. Picture were taken post 7-days salinity stress (0-200mM of NaCl). B. Graphical representation of 7-days salinity stress on root growth of MS1-2. C. Post 14-days salinity stress on MS1-2 line using 0-200mM of sodium chloride. D. Graphical representation of 14-days salinity stress on root growth of MS1-2. Data represent mean ± SE. n = 10.

MS1-2-7 (T3 Generation)

The results show that there is a difference in the root growth in the MS1-2-7 lines compared to the wild-type tobacco plants (Fig. 7). The roots of both the MS1-2-7 and WT tobacco plants showed no significant difference when no sodium chloride was added to the growing medium. The roots of the MS1-2-7 plants compared to the wild-type plants both in 150mM and 200mM sodium chloride showed significant difference (Fig. 7 A and B and SD3 in Appendix C). The difference in root growth was still observed for 14 days exposure (7C, and 7D and SD4 in Appendix C) as in 7 days (7A and 7B) but not as obvious as in Fig. 6. The MS1-2-7 2-7 line did not respond to salinity stress in the same pattern as MS1-2 or MS1-2-14. It suggests that this line might be finding it hard to tolerate the saline condition and balance the ion content in tissues as the length of the exposure to salt increased.

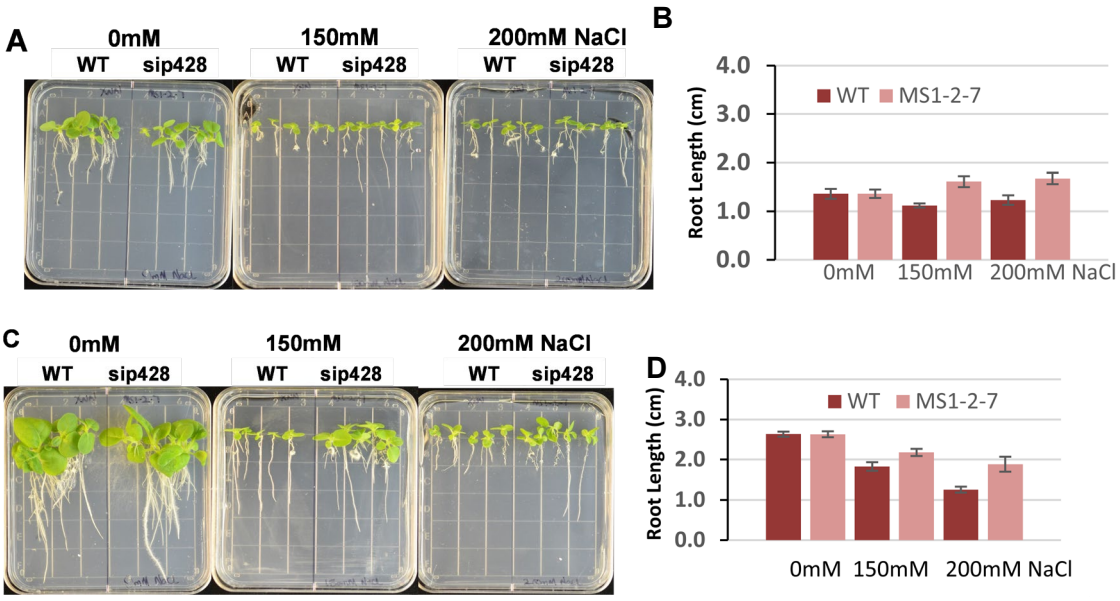

Figure 7. **Root growth of MS1-2-7 sip428-silenced plants under salinity stress**. **A**. Picture were taken post 7-days salinity stress (0-200mM of NaCl). **B**. Graphical representation of 7-days salinity stress on root growth of MS1-2-7. **C**. Post 14-days salinity stress on MS1-2-7 line using 0-200mM of sodium chloride. **D**. Graphical Representation of 14-days salinity stress on root growth of MS1-2-7. Data represent mean ± SE. n = 10.

MS1-2-14 (T3 Generation)

Results show that there is a difference in the root growth in the MS1-2-14 lines compared to the wild-type tobacco plants. There was no significant difference in the roots of both the MS1-2-14 and WT tobacco plants when the growing medium had no sodium chloride. There was significant difference in the roots of the MS1-2-14 compared to the wild-type plants both in 150mM and 200mM sodium chloride concentrations, especially at 200mM sodium chloride (Fig. 8 A and B and SD5 in Appendix C). The difference in root growth was more at 14 days exposure (8C, and 8D and SD6 in Appendix C) compared to the 7 days (8A and 8B). The wild-type plants seem to have had an initial sensitivity to salinity but there was subsequent recovery which is probably due to acclimation. Contrary to the hypothesis, the MS1-2-14 had similar pattern to MS1-2 indicating that they are also not affected by saline conditions and are probably able to exclude the ions from their tissues.

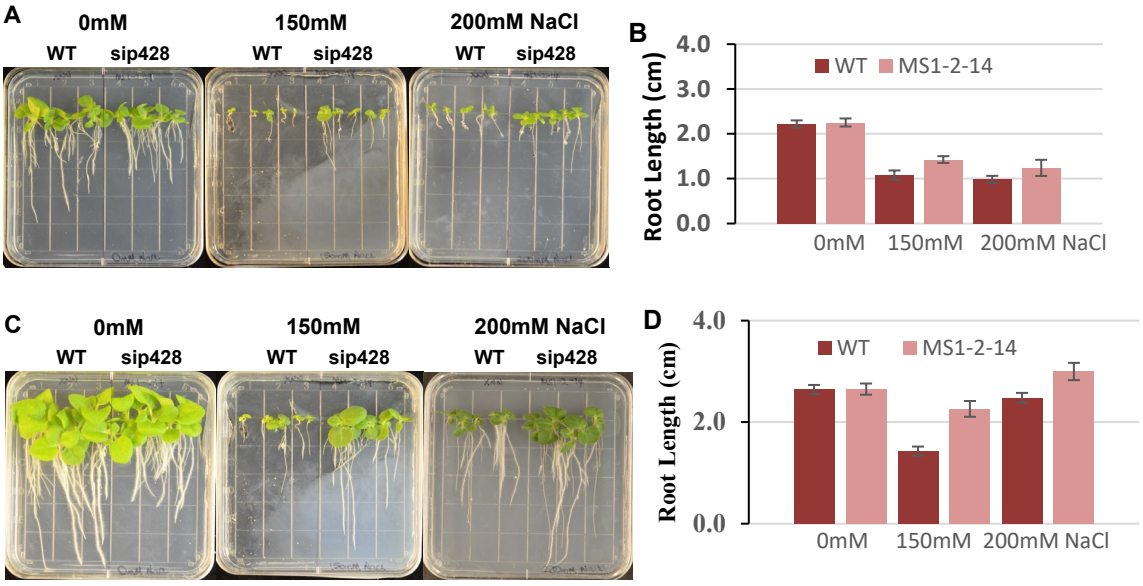

Figure 8. **Root growth of MS1-2-14 sip428-silenced plants under salinity stress**. **A**. Picture were taken post 7-days salinity stress (0-200mM of NaCl). **B**. Graphical representation of 7-days salinity stress on root growth of MS1-2-14. **C**. Post 14-days salinity stress on MS1-2-14 line using 0-200mM of sodium chloride. **D**. Graphical Representation of 14-days salinity stress on root growth of MS1-2-14. Data represent mean ± SE. n = 10.

The onset of salinity stress causes an ionic imbalance in plants and tolerance can only be achieved by maintaining ionic homeostasis (Munns and Tester 2008). The hypothesis was that the sip428-silenced lines would be sensitive to salinity stress and would find it difficult to thrive. In contrast to that, the sip428-silenced lines seem to thrive better under salinity stress (Fig. 6 and Fig. 8). The roots of MS1-2 lines grew longer at 7- and 14-days under 150mM and 200mM concentration NaCl than wild-type (Fig. 6). The MS1-2-7 lines also showed tolerance under both concentrations on both days (Fig. 7) but the effect was not as much as the other lines. MS1-2-14 showed better root growth at both concentrations, more at 200mM but the plants showed sensitivity and subsequent recovery (Fig. 8). Plants differ in their tolerance to stress but the difference in phenotype between the lines is unclear and requires further studies. Mehdi et al. showed that low levels of HDA19 increased salt tolerance in *Arabidopsis thaliana* (Mehdi et al. 2016). Ueda et al. also showed that the HDA19 mutant exhibited increased tolerance to salinity stress (Minoru Ueda et al. 2017). Analysis of the effect of the expression level of AtSRT1 showed that RNAi-mediated silencing led to resistance while wild-type and overexpression lines were susceptible to NaCl and abscisic acid treatment (Liu et al. 2017b). The results presented here show that sip428-silenced lines show reduced sensitivity (or increased tolerance) to NaCl compared to wild-type plants. This suggests that a high level of SIP428 expression might make the wild-type sensitive and that SIP428 might have a negative role in regulating plant stress response. Several genes with functions in detoxification are induced during salinity stress response (Seki et al. 2002). Therefore, stress-responsive genes such as glutathione peroxidase and ABA-responsive element-binding factor expression should be tested. Also, the sip428-overexpression lines should be tested to see what effect salinity stress would have on them. Besides this, the antioxidant activity of antioxidant enzymes such as peroxidase, catalase is

crucial for ROS scavenging and should also be tested. This would give insight into the level of ROS produced and the scavenging activity of the enzymes. Also, SIP428 helps to regulate SABP2, therefore, examining the effect on sip428 silencing on SABP2 activity would help to provide better insight.

Relative Chlorophyll Content of The Wild-Type and Sip428-Silenced Line Post Salt Treatment

Photosynthetic efficiency is a good determinant of the overall health of a plant. The chlorophyll a content in wild-type and MS1-2 sip428-silenced lines after ten days of salinity stress treatment, showed that there is no significant difference in chlorophyll content (Fig. 9A). This result was confirmed by the data from the PhotosynQ device (Fig. 9B).

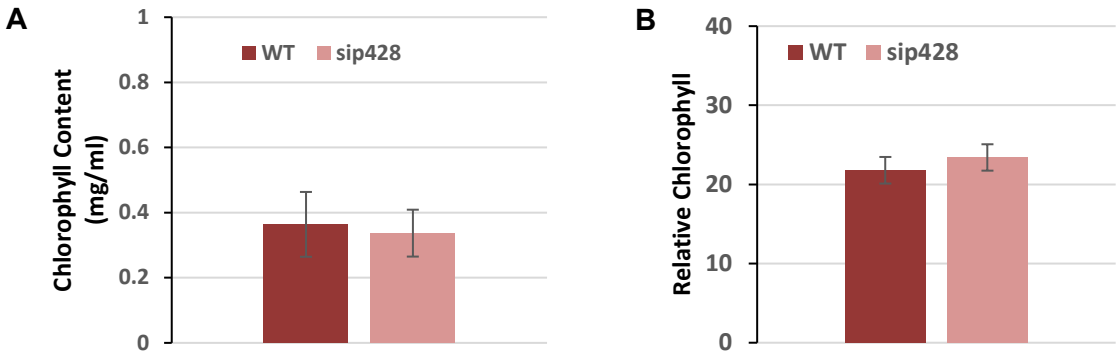

Figure 9. **Chlorophyll a content of MS1-2 sip428-silenced plants under salinity stress.** Chlorophyll content in wild-type and MS1-2 SIP428-silenced lines 10 days post-salinity stress. **A**. Data from chlorophyll a. **B**. Data for total chlorophyll from PhotosynQ. n = 6.

Role of SIP428 in Mediating Osmotic Stress

Osmotic stress can be measured by including mannitol in the growing media. In an initial pilot study to test varying concentrations of mannitol, seeds of sip428-silenced MS1-2 transgenic tobacco plants from the T2 generation were used. Ten-day-old seedlings of both the wild-type

and the MS1-2 lines grown in 1/2x MS media were transferred to the MS media plates containing 0, 100, 200, and 300mM of mannitol to induce osmotic stress. The seedlings were allowed to grow under 12 h of light in a temperature-controlled room (set at 22°C). The growth of the seedlings was observed for 14 days. Results from this experiment showed that both the wild-type and the sip428-silenced lines were sensitive to mannitol at all three concentrations of mannitol compared to the control plate with no mannitol in the growing medium, but the sensitivity was higher at 300mM (Fig. 10). A similar effect was observed when the seedlings were grown to determine the effects of mannitol on the growth of the root (Fig. 11). Therefore, 150mM and 200mM concentration were used for subsequent experiments as the plants were able to grow and leaf expansion occurred at lower concentrations.

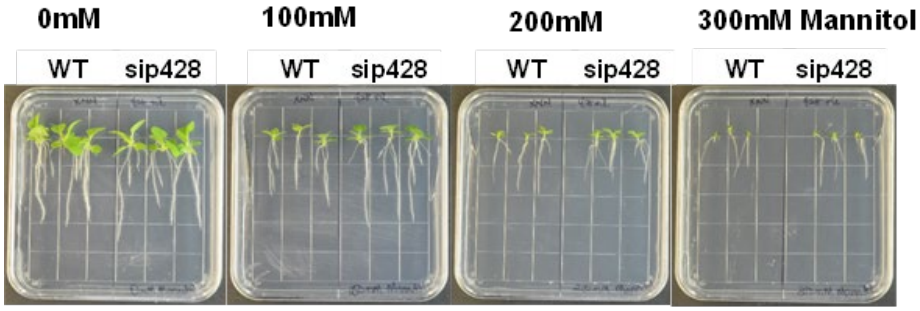

Figure 10. **Growth of MS1-2 sip428-silenced plants under osmotic stress**. Seedlings of WT (wild-type) and sip428 lines were grown in 0,100,200 and 300mM mannitol for 14 days. Experiments were repeated twice with similar results.

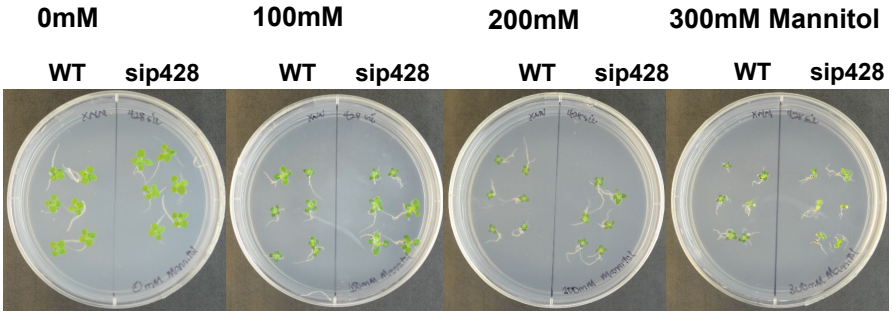

Figure 11. **Growth of roots in MS1-2 sip428-silenced plants under osmotic stress.** Seedlings from this experiment were used to determine the root length. Experiments were repeated twice with similar results.

Comparison Between Three Silenced Lines Exposed to Osmotic Stress.

To compare the effect of plant growth in response to dose-dependent mannitol exposure, three different sip428-silenced lines, MS1-2, MS1-2-7, and MS1-2-14 were used. It is hypothesized that all three lines will respond similarly to the stress caused by the exposure to the mannitol. The sip428-silenced lines were grown on MS media plates containing 0mM, 150mM, and 200mM mannitol (Fig. 12, Fig. 13, and Fig. 14). Although both wild-type and T2 plants showed sensitivity to mannitol, results show that there was a difference in the root growth in the sip428-silenced lines compared to the wild-type tobacco plants.

MS1-2 (T2 Generation)

The roots of both the sip428-silenced and WT tobacco plants showed no significant difference when no mannitol was added to the growing medium and there was no significant difference between them. Results show that seven days after treatment, the wild-type plants exhibited sensitivity to mannitol, and there was a difference in the root growth in the MS1-2 lines compared to the wild-type tobacco plants. The roots of the MS1-2 plants show significant difference compared to the wild-type plants both in 150 mM and 200 mM mannitol treated seedlings (Fig. 12 A and B and SD7 in Appendix C). The difference in root growth was significant upon 14 days exposure for 150mM concentration (12C and 12D and SD8 in Appendix C) than 200mM concentration (12A and 12C). The response of the MS1-2 line shows that the line was less sensitive (more tolerant) than the wild-type plant. This result did not support the hypothesis and a plausible explanation would be that there is a reduced rate of transpiration in the sip428-silenced MS1-2 line. This should be tested.

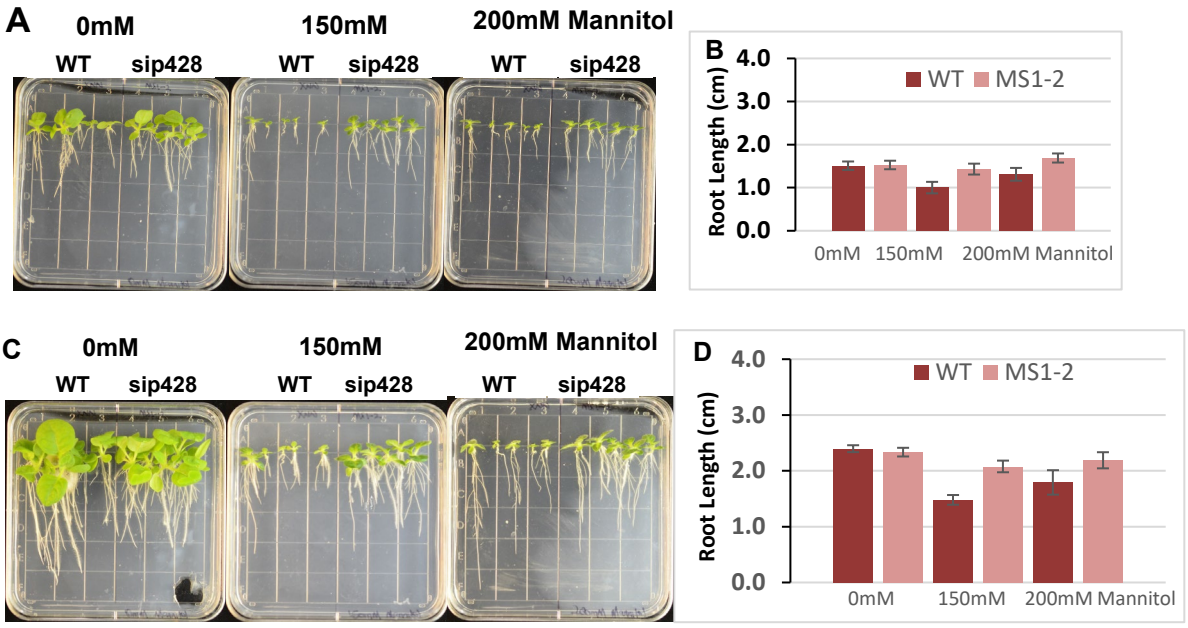

Figure 12. **Root growth of MS1-2 sip428-silenced plants under osmotic stress**. **A**. Pictures were taken post 7-days osmotic stress (0 - 200mM of mannitol). **B**. Graphical representation of 7-days osmotic stress on root growth of MS1-2. **C**. Post 14-days osmotic stress on MS1-2 line using 0-200mM of mannitol. **D.** Graphical representation of the effect of mannitol on root growth in MS1-2. Data represent mean ± SE. n = 10.

MS1-2-7 (T3 Generation)

Results show that seven days after treatment, the plants exhibited sensitivity to mannitol, and there is a difference in the root growth in the sip428-silenced lines compared to the wild-type tobacco plants. The roots of both the MS1-2-7 and WT tobacco plants showed no significant difference when no mannitol was added to the growing medium. There was significant difference in roots of the MS1-2-7 plants compared to the wild-type plants both in 150 mM and 200 mM mannitol (Fig. 13 A and B and SD9 in Appendix C). The difference in root growth was more significant at 14 days post exposure in 200mM mannitol (13C and 13D and SD10 in Appendix C) than at 7 days (13A and 13B). This suggests that the initial onset of the stress might have induced stress responsive genes which enabled recovery after 14 days. The pattern observed in MS1-2-7 is similar to that of MS1-2 but the MS1-2-7 seems to have a better leaf expansion indicating they might have better water use efficiency.

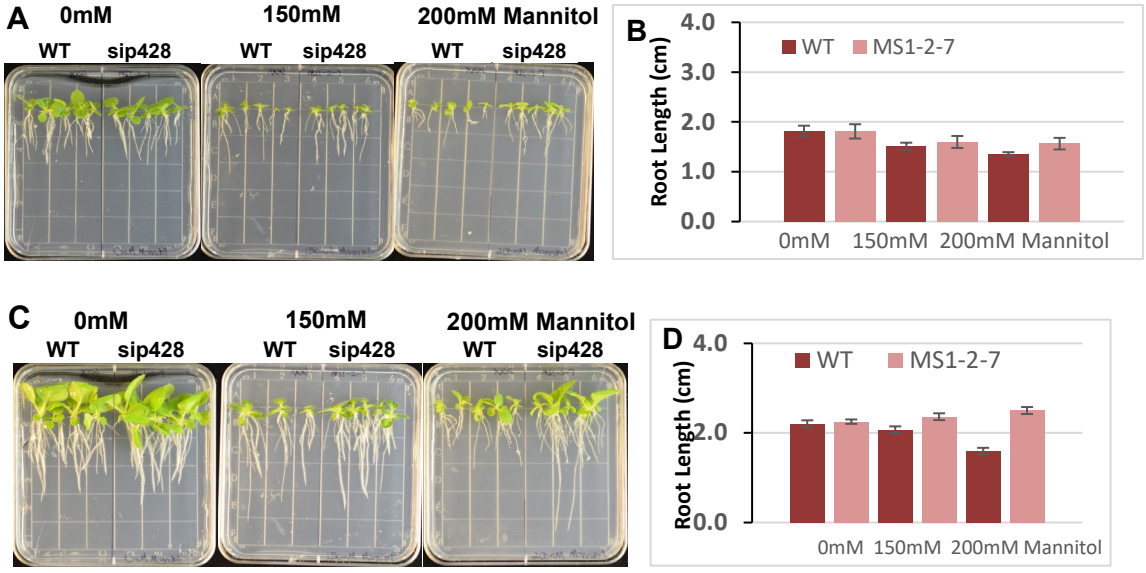

Figure 13. **Root growth of MS1-2-7 sip428-silenced plants under osmotic stress. A**. Pictures were taken post 7-days osmotic stress (0 - 200mM of mannitol). **B**. Graphical representation of 7-days osmotic stress on root growth of MS1-2-7. **C**. Post 14-days osmotic stress on MS1-2-7 line using 0-200mM of mannitol. **D**. Graphical representation of the effect of mannitol on root growth in MS1-2-7. Data represent mean ± SE. n = 10.

MS1-2-14 (T3 Generation)

Results show that there is a difference in the root growth in the MS1-2-14 lines compared to the wild-type tobacco plants at higher mannitol concentrations. The roots of both the MS1-2-14 and WT tobacco plants showed no significant difference when no mannitol was added to the growing medium There was significant difference in the roots of the MS1-2-14 plants in 150mM compared to the wild-type plants in 200 mM mannitol (Fig. 14 A and B and SD11 in Appendix C). The difference in root growth in 150mM mannitol was observed at 14 days after exposure (14C, and 14D and SD12 in Appendix C). The response of MS1-2-14 was different from that of MS1-2 and MS1-2-7, especially at 200mM with both wild-type and MS1-2-14 showing sensitivity. It is possible that the plants are unable to undergo osmotic adjustment after prolonged exposure.

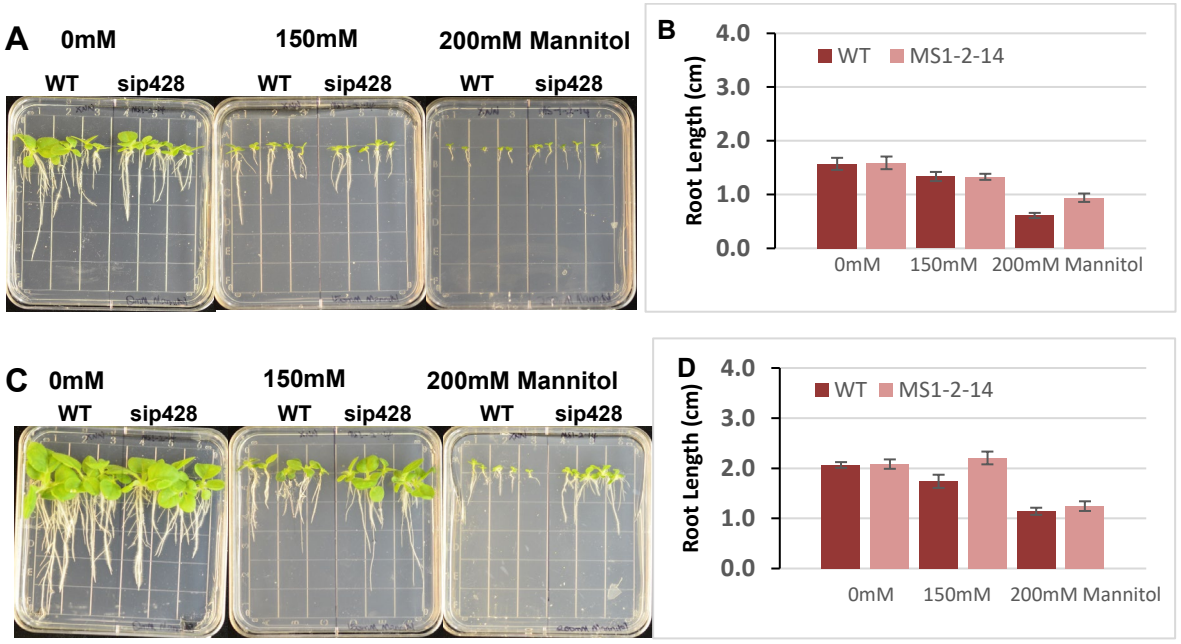

Figure 14. **Root growth of MS1-2-14 sip428-silenced plants under osmotic stress**. **A**. Pictures were taken post 7-days osmotic stress (0 - 200mM of mannitol). **B**. Graphical representation of 7-days osmotic stress on root growth of MS1-2-14. C. Post 14-days osmotic stress on MS1-2-14 line using 0-200mM of mannitol. **D**. Graphical representation of the effect of mannitol on root growth in MS1-2-14. Data represent mean ± SE. n = 10.

Osmoticum such as mannitol can induce stress in plants by reducing the water potential of the synthetic media making it difficult for the plant to utilize water. Osmotic stress causes suppression of photosynthesis. The hypothesis was that the sip428-silenced lines would be more susceptible than the wild-type under osmotic stress. Results show that MS1-2 had significant root growth in 150mM and 200mM mannitol than wild-type plants. The MS1-2-7 lines showed that the difference between the wild-type and the sip428 silenced line in 150mM mannitol was not significant and while the difference was significant at 200mM concentration (Fig. 13). The MS1-2-14 show that onset of the stress did not have an effect at 150mM concentration compared to 200mM (Fig. 14A), 14-day results show that the silenced lines are able to adapt and thrive in 150mM mannitol than at 200mM (Fig. 14C). Liu et al. also showed that treating *Arabidopsis thaliana* AtSRT1 knockdown lines with mannitol made them tolerant while overexpression lines and wild-type were hypersensitive suggesting a negative role for AtSRT1 in abiotic stress (Liu et al. 2017b). It is likely that the sip428-silenced lines are undergoing better osmotic adjustment than the wild-type to prevent dehydration or they are accumulating sugars or organic acids to help reduce the rate of energy metabolism. AtSRT2 knockout lines had reduced levels of sugar but increased levels of amino acid suggesting that this might be the link to the reduction in energy metabolism exhibited (König et al. 2014). Also, it is possible that the silenced lines are negative regulators in plant defense helping in homeostasis and that the absence of its deacetylase activity aids protein such as the uncoupling protein to help maintain energy status. Analysis to test ADP levels should be done in the knockdown lines. SIP428 overexpression lines would need to also be tested.

Relative Chlorophyll Content of Wild-Type And sip428-Silenced Line Post Mannitol Treatment

The chlorophyll a content in wild-type and MS1-2 sip428-silenced line was compared after ten days of osmotic stress treatment, and the results in Fig. 15 show that there is no significant difference in chlorophyll a content (Fig. 15A). This was further confirmed by the data from the PhotosynQ device (Fig. 15B).

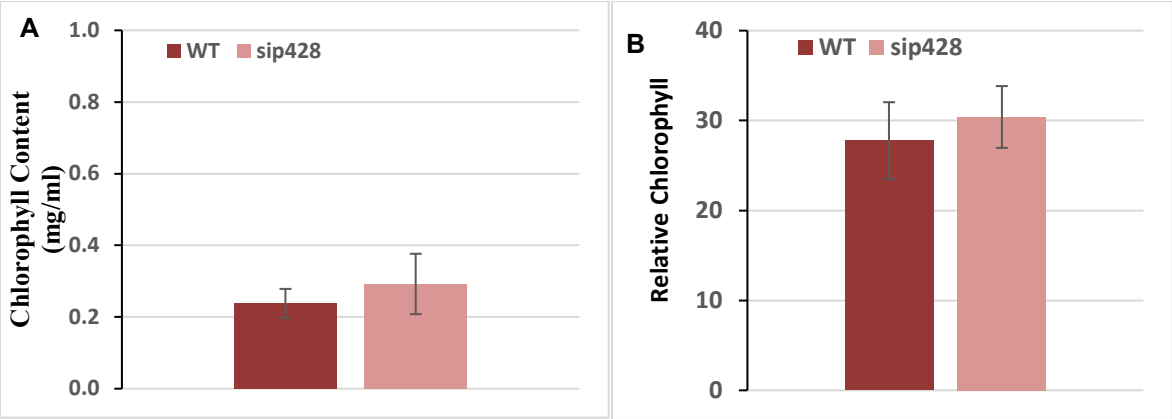

Figure 15. **Chlorophyll a content of MS1-2 sip428-silenced plants under osmotic stress.** Chlorophyll content in wild-type and SIP428 silenced lines under osmotic stress. **A**. Data from chlorophyll a. **B**. Data for total chlorophyll from PhotosynQ (right). n = 6

Role of SIP428 in Mediating Drought Stress

Drought stress can be measured by including PEG in the growing media. In an initial pilot study to test varying concentrations of drought-inducing chemical, polyethylene glycol (PEG) 8000, wild-type and MS1-2 sip428-silenced tobacco from the T2 generation were used. Ten-day-old seedlings of both the wild-type and the MS1-2 lines grown in 1/2x MS media were transferred to the 1/2x MS media plates containing 0, 1%, 3%, and 5% PEG to induce drought. The seedlings were allowed to grow under 12 h of light in a temperature-controlled room. The growth of the seedlings was observed and recorded for 14 days. Results from this experiment

showed that the PEG concentration of 1% did not have as much effect compared to the other concentrations (3 and 5%) for both the wild-type and the sip428-silenced MS1-2 tobacco seedlings (Fig. 16). A similar effect was observed when the seedlings were grown to determine the effects of PEG on the growth of the root (Fig. 17). Therefore, 3% and 5% concentrations were used for further experiments.

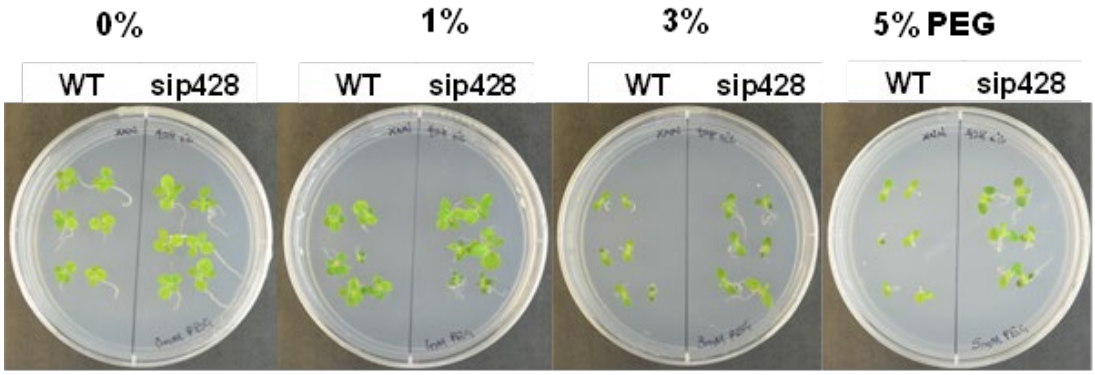

Figure 16. **Growth of MS1-2 sip428-silenced plants under drought stress**. Seedlings of WT (wild-type) and sip428 lines were grown in 0,1,3 and 5% polyethylene glycol for 14 days. Experiments were repeated twice with similar results.

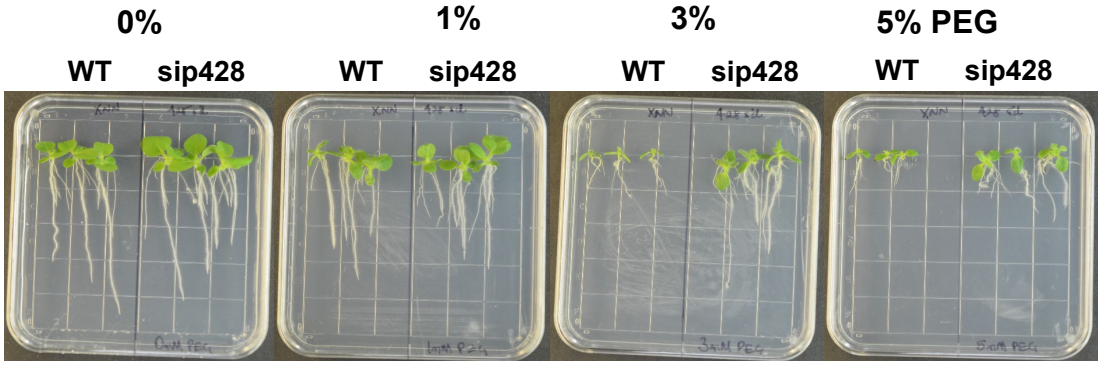

Figure 17. **Growth of roots in MS1-2 sip428-silenced plants under drought stress.** Seedlings from this experiment were used to determine the root length. Experiments were repeated twice with similar results.

Comparison Between Three Silenced Lines Exposed to Drought Stress.

To compare the effect of plant growth in response to drought stress in three different sip428-silenced lines, MS1-2, MS1-2-7, and MS1-2-14, varying amounts of PEG were added to the media. It was hypothesized that all three lines will produce similar visible phenotypes upon exposure to different concentrations of PEG. This was done to observe the impact of these concentrations on the root growth of the seedlings.

MS1-2(T2 Generation)

The wild-type and MS1-2 seedlings were grown on plates containing 3% and 5% polyethylene glycol (Fig. 18). Results show that there was significant difference in the root growth in the MS1-2 lines compared to the wild-type tobacco plants. There was no significant difference in growth of root of both the MS1-2 and WT tobacco plants when no polyethylene glycol was added to the growing medium. The roots of the MS1-2 plants grew longer compared to the wild-type plants both in 3% and 5% polyethylene glycol (Fig. 18A and B and SD13 in Appendix C). The difference in root growth was as significant upon 14 days exposure (18C and 18D and SD14 in Appendix C) as 7days (18A and 18B). The response of MS1-2 lines suggests that the plants were not sensitive. Conversely, it did not support the hypothesis. It is likely that stomatal aperture is under the control of signals linked to stress response to reduce water loss.

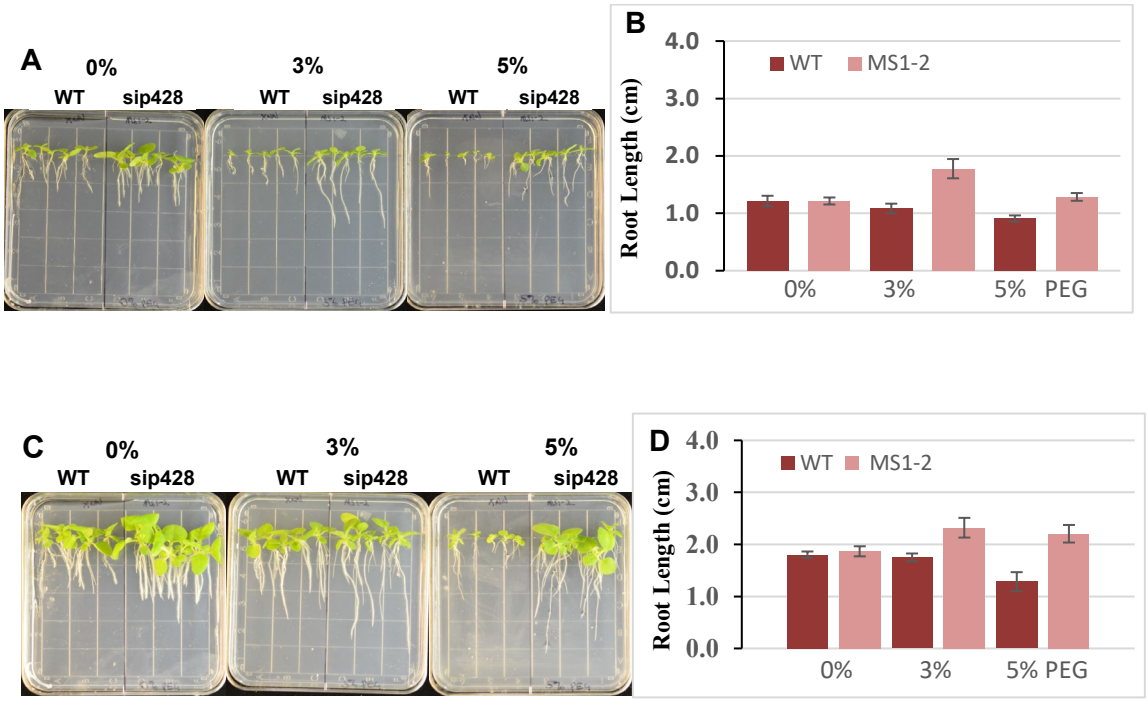

Figure 18. **Root growth of MS1-2 sip428-silenced plants under drought stress. A**. Post 7-days drought stress on MS1-2 line using 0-5% of PEG. **B**. Graphical representation of the root length post 7-days of drought stress. **C**. Post 14-days drought stress on MS1-2 line using 0-5% of polyethylene glycol. **D**. Graphical representation of the root length. Data represent mean ± SE. n = 10.

MS1-2-7 (T3 Generation)

Results show that there is a difference in the root growth in the MS1-2-7 lines compared to the wild-type tobacco plants. There was no significant difference in the roots of both the MS1-2-7 and WT tobacco plants when no polyethylene glycol was added to the growing medium. The difference in the roots of the MS1-2-7 plants was statistically significant compared to the wild-type plants both in 3% and 5% polyethylene glycol (Fig. 19A and B and SD15 in Appendix C). The difference in root growth was more significant in 3% after 14 days exposure (19C and 19D and SD16 in Appendix C) than 7days (19A and 19B). It showed that the initial exposure affected the wild-type but they recovered after 14 days. Contradictory to the hypothesis, the MS1-2-7 were not outrightly sensitive. They showed more of the sensitivity at 5% and not 3% after 14 days and they did not have the same pattern as MS1-2 or MS1-2-14 which thrived in 3% and 5%

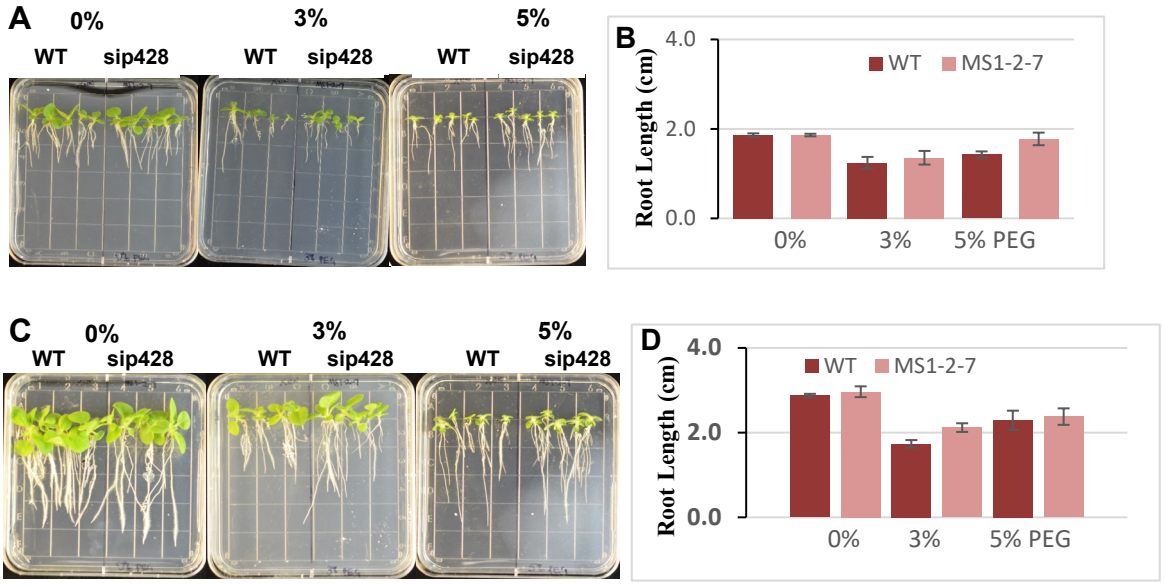

Figure 19. **Root growth of MS1-2-7 sip428-silenced plants under drought stress. A**. Post 7-days drought stress on MS1-2-7 line using 0-5% of PEG. **B**. Graphical representation of the root length post 7-days of drought stress. **C**. Post 14-days drought stress on MS1-2-7 line using 0-5% of polyethylene glycol. **D**. Graphical representation of the root length. Data represent mean ± SE. n = 10.

PEG. The effect observed might be due to accumulation of organic osmolyte which enable them to survive the stress but alters their growth.

MS1-2-14 (T3 Generation)

Results show that there is a difference in the root growth in the MS1-2-14 sip428-silenced lines compared to the wild-type tobacco plants. There was no significant difference in the roots of both the MS1-2-14 and WT tobacco plants when no polyethylene glycol was added to the growing medium. The roots of the MS1-2-14 plants showed significant difference compared to the wild-type plants both in 3% and 5% polyethylene glycol (Fig. 20A and B and SD17 in Appendix C). The difference in root growth was more pronounced upon 14 days exposure (20C and 20D and SD18 in Appendix C) than 7days (20A and 20B) especially for 5%.

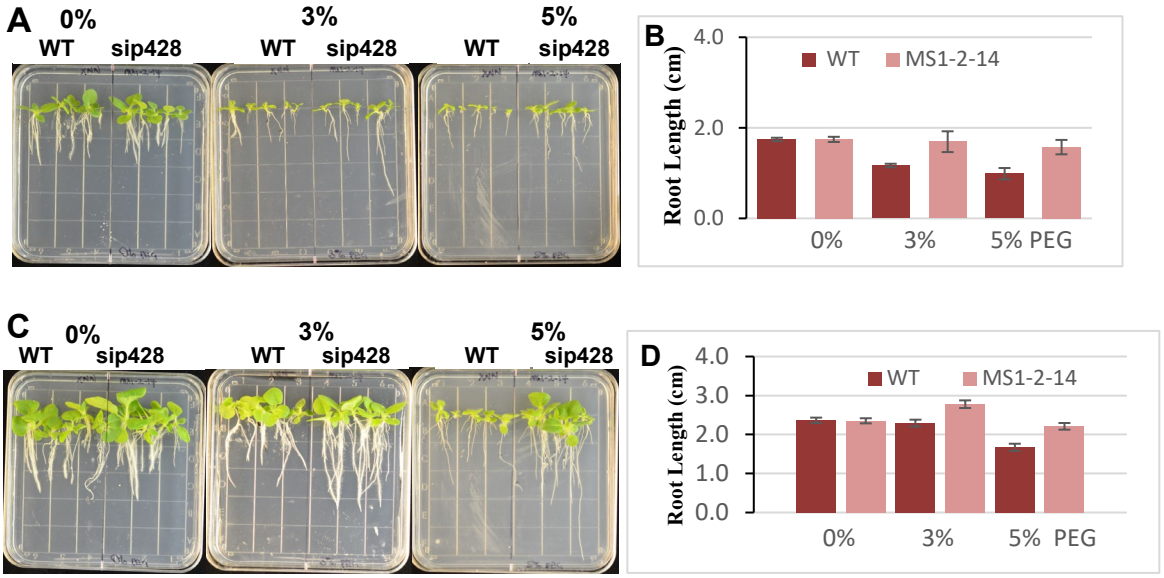

Figure 20. **Root growth of MS1-2-14 sip428-silenced plants under drought stress. A**. Post 7-days drought stress on MS1-2-14 line using 0-5% of PEG. **B**. Graphical representation of the root length post 7-days of drought stress. **C**. Post 14-days drought stress on MS1-2-14 line using 0-5% of polyethylene glycol. **D**. Graphical representation of the root length. Data represent mean ± SE. n = 10.

The MS1-2-14 show similar pattern of growth in 150mM mannitol, and similar pattern of growth to MS1-2. This suggests that there might be a mechanism used by these plants to sense and restore normal processes under drought stress.

Drought stress hinders root growth. Adjustment of plants to water-deficit conditions requires mechanisms to optimize water uptake . Results from this study show that the knockdown of SIP428 helps protects tobacco plants. The MS1-2 sip428-silenced lines had more root growth in 3% PEG at the onset of stress than the wild-type (Fig. 18). The MS1-2-7 sip428-silenced lines showed a slight difference at 3% but not 5% (Fig. 19). The MS1-2-14 sip428-silenced lines show tolerance to 3% than 5% (Fig. 20). Therefore, it shows that drought stress affects the wild-type and not the sip428-silenced lines. Studies on HDA9 mutants show that its deficiency in Arabidopsis made the plant tolerant to drought stress with a highly significant difference in root length and germination rates after 5days compared to wild-type (Zheng et al. 2016). Ueda et al. showed that HDA19 deficiency in Arabidopsis increased its tolerance to drought (Ueda et al. 2018). It is likely that stress defense genes are being activated during the initial stages of exposure that gives the sip428 knockdown lines this protection since their induction can help protect the plants. It would be important to conduct experiments to analyze the expression of different stress defense genes or transcription factors such as (dehydration-responsive element-binding protein) DREB, ABA-dependent transcription factor, MYC2. Also, the water use efficiency of the sip428-silenced lines should be analyzed to understand if they are losing or utilizing water more than the wild-type or overexpression lines. There is a need to repeat this experiment and analyze sip428 transcripts to further understand the difference observed in the lines. There is also a need to visualize the stomata of the plants before and after stress.

Relative Chlorophyll Content of Wild-Type and SIP428-Silenced Line Post-PEG Treatment

The relative chlorophyll content in wild-type and MS1-2 SIP428-silenced line was measured after two weeks PEG treatment, and the result (Fig. 21) show that there is no significant difference in chlorophyll content (Fig. 21A). This was confirmed by the data from the PhotosynQ device (Fig. 21B).

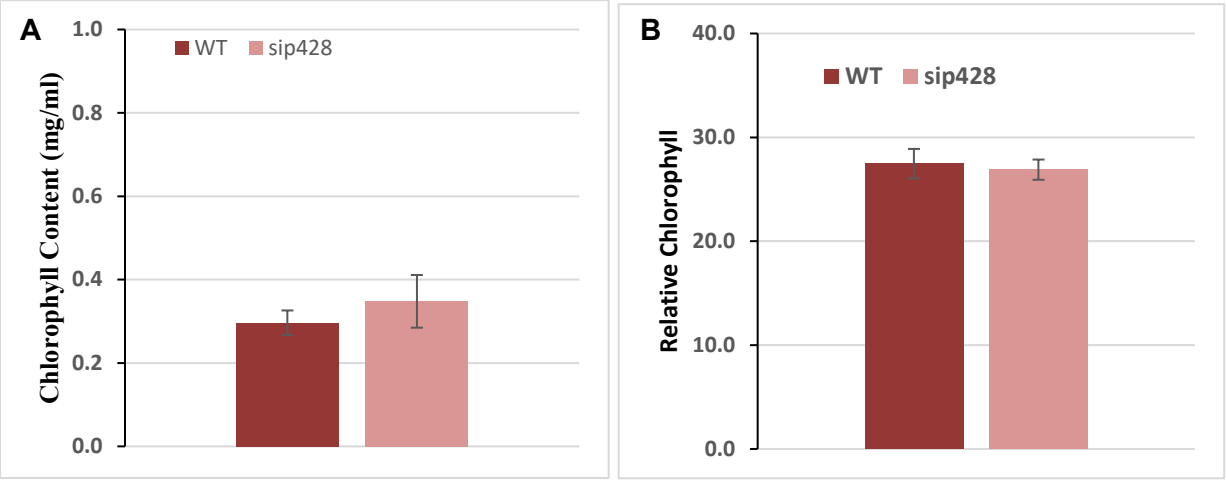

Figure 21. **Chlorophyll a content of MS1-2 SIP428-silenced plants under drought stress.** Chlorophyll content in wild-type and MS1-2 sip428 silenced line under drought stress. **A**. Data from chlorophyll a (left), **B**. Data of total chlorophyll from PhotosynQ (right). n = 6

CHAPTER 4. SUMMARY AND FUTURE DIRECTIONS

Environmental stresses in plants such as drought, and salinity can induce osmotic stress (Xiong and Zhu 2002). Salinity is a key restricting factor of plant growth and yield (Allakhverdiev et al. 2000). Responding to environmental stresses, many plant proteins undergo regulation which often enables plants to detoxify toxins, restore homeostasis and recovery (Xiong and Zhu 2002). These results in increased level of reactive oxygen species in organelles leading to oxidative damage (Chutipaijit 2016). To test the effect of these salinity, osmotic and drought stress in synthetic media, various concentrations of each of the stress-inducing chemicals were initially used, and later, only specific concentrations were used for further testing. The rationale was that the effect was more obvious at intermediate concentrations compared to the higher concentrations that were toxic and significantly hindered the growth of both the WT and the sip428-silenced lines.

Abiotic stresses affect crop yield as well as biochemical processes in plants (Cheng et al. 2013). In healthy plants, water is crucial for maintaining homeostasis and growth. Exposure of plants to oxidative and osmotic stress alters water balance which in turn alters growth. Therefore, a crucial morphological and developmental effect of stress is the change in growth rate. To assess stress in plants, visible stress phenotypes such as root length is used. Root length is a marker of tolerance as longer roots can help plants absorb more water and increase biomass under water-deficit conditions (Wasson et al. 2012). Results from the root length experiment show that for salinity and drought stress, there is a significant difference exhibited by the sip428-silenced transgenic lines when compared to the wild-type tobacco plants. Contrary to the hypothesis, the sip428-silenced transgenic tobacco plants showed more root growth compared to wild-type tobacco plants over a two week period. This study suggests that knockdown of SIP428

may protect the plants against abiotic stress. It showed that SIP428 might be a negative regulator in plant stress response as knockdown of SIP428 increased tolerance of tobacco plants under stress. Therefore, an increased level of SIP428 would not provide tolerance in plant.

The link between abiotic stresses and photosynthesis is that when the stress occurs, the levels of reactive oxygen species are elevated, stomata close, a decrease in carbon dioxide diffusion from the atmosphere into the leaves occurs, and these processes likely reduces the overall rate of photosynthesis (Muhammad et al. 2021). Reports from previous studies of abiotic stresses show that during the onset and progression of salinity stress, major plant processes such as photosynthesis, and energy metabolism are affected (Parida and Das 2005). The data from relative chlorophyll content in sip428-silenced plants suggests that photosynthetic efficiency was not significantly affected despite the exposure of these transgenic plants to salinity, osmotic, or drought stress. Since the chlorophyll a content was analyzed only once, there is a need to repeat this experiment with a larger sample size to confirm if photosynthesis is affected or not. Although, König et al showed that ATP levels remained unchanged in AtSRT2 knockout plants suggesting that the plants were photosynthesizing (König et al. 2014).

The role of SA in abiotic stress tolerance and antioxidant systems has been studied (Borsani et al. 2001). It has been shown that SA induces tolerance to salinity stress by enhancing the antioxidative systems which help to activate photosynthesis and alleviate stress (Li, Hu, et al. 2014; Ma et al. 2017). SA has also been shown to help in modulating enzymes that strengthen antioxidant systems in plants subjected to drought stress (Saglam et al. 2012). Levels of SA accumulation also increases during stress (Munné-Bosch & Peñuelas 2003). SIP428 which is an SABP2-interacting protein, is thought to play a role in regulating SABP2 levels in plants. This study shows that the knockdown of SIP428 had no visible effect on the morphology, but its

absence might have a yet to be ascertained implication on the SA-signaling. The question therefore arises that, what is the mechanism being used by the sip428-silenced transgenic plants to improve its tolerance? Could it be that they have better antioxidative activity? It is possible that the plants have developed a way to adapt to its absence or that its accumulation is not required in plant response. Also, a few SABPs such as α-ketoglutarate dehydrogenase, and glyceraldehyde phosphate dehydrogenase are directly involved in the energy metabolism, and have been identified to be acetylated (Wang et al. 2010; Liu, et al. 2014).

All three lines used in this study, are silenced in SIP428 expression, it was expected that all three lines would exhibit similar phenotypes upon exposure to stress inducing chemicals. Contrary to this, there was difference in response between T2 and T3 generations to these various stressors. Also, there was observable difference between the T3 generations. This could be because of the level of SIP428 expression in each of these lines.

Overall, the analysis of the sip-silenced lines has shown that the absence of SIP428 did not negatively affect the physiology of the plants. The sip428-silenced lines did show enhanced tolerance under all the three stresses tested. It is possible that abiotic stress tolerance is conferred by controlling cellular energy metabolism. If this hypothesis holds true that SIP428 helps in energy metabolism, then SIP428 probably works in regulating an uncoupling protein of the respiratory chain in the mitochondria which helps in the plants' adaptation to these stresses. (König et al. 2014) have shown, using the *Arabidopsis* sirtuins which have high homology with SIP428, has functions in energy metabolism interacting with crucial proteins in the mitochondria. It is also worthy of note that certain sirtuins could be induced during nutrient shortage as a survival mechanism as postulated by studies in caloric restriction (Cohen et al. 2004).

Reactive oxygen species (ROS) are produced when plants are exposed to stress which can damage the cell, pigments, membrane, and eventually lead to cell death (Simova-Stoilova et al. 2008). It is thought that restriction of electron flow because of the exposure to the different stressors ultimately leads to ROS production (Chutipaijit 2016). Biochemical analysis should be done on sip428-silenced lines to test the level of antioxidant enzyme activity. The enzyme activity that should be tested should include catalase (CAT), superoxide dismutase (SOD), and peroxidase (POX). This will help support the observation that the sip428-silenced transgenics show enhanced tolerance to various abiotic stress. Several studies have demonstrated that stress conditions induce the expression of sirtuins and that sirtuins help in regulating transcription factors that induce the antioxidant response of SOD and POX (Anna et al. 2005; Tseng et al. 2013).

Sirtuins' presence in other plant species suggests that they may have importance in growth and response to stress. One of the crucial next steps is to also identify the subcellular localization of the SIP428. This would enable careful examination in relation to an adaptation of SIP428 and the energy status could also be examined by quantifying the ATP/ADP ratio. This would give a better understanding of this enzyme and its likely function. Then, there is a need to identify the acetylome, to know about the acetylated proteins in tobacco plants that are being deacetylated by SIP428. Essentially, for sirtuins that have already been studied so far, it has been shown to help in the regulation of homeostasis in the nucleus or mitochondria depending on their localization. These findings require further investigation to enable us to understand the role of SIP428 in plant physiology. There is a need to repeat these experiments and conduct transcript analysis to evaluate the expression of SIP428 and some other stress-responsive genes.

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APPENDICES

Appendix A: Abbreviations

SABP2 - Salicylic acid binding protein 2

SIP428 – SABP2 Interacting Protein-428

XNN - Wild-type plants (Nicotiana tabacum cv Xanthi nc),

SA - Salicylic acid

JA - Jasmonic acid

SAR - Systemic acquired resistance

MeSA - Methyl salicylate

ROS - Reactive oxygen species

SOD- Superoxide dismutase

PEG- Polyethylene glycol

NaCl- Sodium chloride

PAD4 - Phytoalexin Deficient

µl - micro litre

ml - milli litre

mM - milli Molar

Appendix B: Buffers and Reagents

MS media with Gamborg's Vitamins

MS media (2.2g/L)

Sucrose (10g/L)

Phytoagar (8g/L)

Myo-Inositol (100mg/L)

Nicotinic Acid (1mg/L)

Pyrodoxine. HCl (1mg/ml)

Thiamine. HCl (10mg/ml)

80% Acetone (100ml)

Acetone (80ml)

Add volume to 100ml with distilled water.

Appendix C: Supplemental Data

Ten plants were used to ascertain root length for each of the treatment groups. The dataset shows the mean, standard deviation and standard error used for the graphical representation.

SALINITY STRESS

SD1:

Day 7	Salt					
	0mM		150mM		200mM	
Plant #	MS1-2	WT	MS1-2	WT	MS1-2	WT
1	1.415	1.315	2.634	2.916	2.082	1.494
2	1.065	1.53	1.736	0.925	1.669	1.216
3	1.58	1.717	2.229	1.665	2.697	2.131
4	1.463	1.206	1.457	1.223	2.247	0.895
5	1.668	1.604	1.518	1.749	2.317	2.694
6	1.145	0.709	1.546	1.074	1.144	1.05
7	1.15	1.213	1.39	1.098	1.097	0.881
8	0.719	1.111	1.14	1.194	1.071	1.039
9	0.789	0.803	1.272	0.733	0.834	0.912
10	1.309	1.112	1.237	0.909	0.811	0.839
MEAN	1.2303	1.232	1.6159	1.3486	1.5969	1.3151
SD	0.317201	0.326079	0.4721	0.636545	0.693074	0.624454
SE	0.100308	0.103115	0.149291	0.201293	0.219169	0.19747

SD1: Ten plants each of wild-type and MS1-2 SIP428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0, 150 and 200mM) of sodium chloride and corresponding value for each root length at 7days.

SD2:

	Day 14					
	Salt					
	0mM		150mM		200mM	
Plant #	MS1-2	WT	MS1-2	WT	MS1-2	WT
1	2.647	3.032	3.858	2.569	3.198	2.846
2	2.944	2.713	2.605	2.514	3.033	2.208
3	2.636	3.204	2.688	1.711	3.339	2.003
4	2.39	2.849	2.776	2.085	2.835	2.138
5	2.412	2.543	2.761	1.988	2.612	2.207
6	2.418	2.319	2.517	1.797	3.037	2.429
7	3.371	2.581	2.437	2.285	3.823	2.222
8	3.104	2.674	2.824	1.958	2.635	2.457
9	2.307	2.478	2.006	1.59	3.141	2.453
10	2.401	2.331	2.974	2.601	3.999	2.348
MEAN	2.663	2.6724	2.7446	2.1098	3.1652	2.3311
SD	0.36034	0.2885	0.47311	0.36694	0.45824	0.23407
SE	0.11395	0.09123	0.14961	0.11604	0.14491	0.07402

SD2: Ten plants each of wild-type and MS1-2 sip428-silenced lines were used for root length data collection. Data show root length in different concentrations (0, 150 and 200mM) of sodium chloride and corresponding value for each root length at 14days.

SD3:

Day 7	Salt					
	0mM		150mM		200mM	
Plant #	MS1-2-7	WT	MS1-2-7	WT	MS1-2-7	WT
1	1.054	1.68	1.433	1.153	1.7	1.482
2	1.664	1.493	2.22	1.111	2.121	1.456
3	1.781	1.868	2.136	1.396	2.083	1.804

4	1.409	1.633	1.698	1.015	2.241	0.92
5	1.577	1.194	1.722	1.168	1.577	1.228
6	1.544	0.823	1.184	1.118	1.407	1.039
7	0.956	1.422	1.343	0.922	1.241	1.292
8	1.132	1.281	1.719	1.134	1.761	1.368
9	1.275	1.024	1.404	0.968	1.363	0.82
10	1.232	1.191	1.256	1.215	1.273	0.913
MEAN	1.3624	1.3609	1.6115	1.12	1.6767	1.2322
SD	0.27596	0.31975	0.35477	0.13425	0.36855	0.31057
SE	0.08727	0.10111	0.11219	0.04245	0.11655	0.09821

SD3: Ten plants each of wild-type and MS1-2-7 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0, 150 and 200mM) of sodium chloride and corresponding value for each root length at 7 days.

SD4:

	Salt					
	0mM		150mM		200mM	
Plant #	MS1-2-7	WT	MS1-2-7	WT	MS1-2-7	WT
1	2.483	2.925	2.335	1.924	2.011	1.193
2	2.726	2.747	2.548	1.695	1.284	1.06
3	2.636	2.301	1.949	1.547	1.53	1.236
4	2.397	2.745	1.61	2.418	1.012	0.991
5	2.831	2.621	1.839	2.026	1.142	1.018
6	2.874	2.407	2.312	1.784	2.329	1.186
7	2.967	2.594	2.344	1.335	2.364	1.771
8	2.582	2.898	2.291	1.432	2.658	1.432
9	2.62	2.571	2.303	1.956	2.198	1.413
10	2.186	2.529	2.25	2.154	2.318	1.291
MEAN	2.6302	2.6338	2.1781	1.8271	1.8846	1.2591
SD	0.23528	0.19962	0.28492	0.33626	0.58943	0.23488

SE	0.0744	0.06313	0.0901	0.10633	0.18639	0.07428

SD4: Ten plants each of wild-type and MS1-2-7 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0, 150 and 200mM) of sodium chloride and corresponding value for each root length at 14 days.

SD5:

	Salt					
	0mM		150mM		200mM	
Plant #	MS1-2-14	WT	MS1-2-14	WT	MS1-2-14	WT
1	2.668	2.356	1.971	0.944	1.079	1.131
2	2.412	2.282	1.547	1.658	1.76	0.886
3	1.828	2.738	1.2	1.007	1.104	1.323
4	2.437	2.233	1.474	0.866	1.645	1.311
5	2.589	1.951	1.129	0.874	2.453	1.15
6	2.13	2.373	1.264	1.351	1.155	0.774
7	2.35	2.032	1.371	1.151	0.668	0.733
8	1.88	1.832	1.563	0.507	0.501	0.593
9	2.162	2.285	1.308	1.411	1.009	0.955
10	2.079	2.113	1.44	1.033	1.039	1.001
MEAN	2.2535	2.2195	1.4267	1.0802	1.2413	0.9857
SD	0.28423	0.25564	0.23931	0.3274	0.57004	0.24533
SE	0.08988	0.08084	0.07568	0.10353	0.18026	0.07758

SD5:Ten plants each of wild-type and MS1-2-14 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0, 150 and 200mM) of sodium chloride and corresponding value for each root length at 7 days.

SD6:

	Salt					
	0mM		150mM		200mM	
Plant #	MS1-2-14	WT	MS1-2-14	WT	MS1-2-14	WT
1	2.885	2.727	1.799	1.299	3.735	3.153
2	2.122	2.749	2.675	1.297	2.705	2.69

3	2.61	3.074	3.168	1.743	3.218	2.506
4	2.878	2.771	2.402	1.641	3.571	2.231
5	2.42	2.354	2.021	1.486	3.649	2.693
6	3.289	3.022	2.605	1.912	3.339	2.481
7	2.398	2.491	2.272	1.519	2.324	2.108
8	2.975	2.662	2.335	1.172	2.238	2.305
9	2.541	2.338	1.833	1.23	2.548	2.254
10	2.391	2.235	1.503	1.004	2.653	2.379
MEAN	2.6509	2.6423	2.2613	1.4303	2.998	2.48
SD	0.34911	0.28491	0.49065	0.28063	0.53844	0.30466
SE	0.1104	0.0901	0.15516	0.08874	0.17027	0.09634

SD6:Ten plants each of wild-type and MS1-2-14 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0, 150 and 200mM) of sodium chloride and corresponding value for each root length at 14 days.

OSMOTIC STRESS

SD7:

	Man					
	0mM		150mM		200mM	
Plant #	MS1-2	WT	MS1-2	WT	MS1-2	WT
1	2.011	2.005	1.464	2.005	1.994	2.233
2	1.266	1.769	1.799	0.782	2.078	0.963
3	2.008	1.909	2.281	1.013	2.197	2.008
4	1.749	1.395	1.386	1.192	1.385	1.092
5	1.274	1.639	1.523	1.316	1.537	1.525
6	1.522	1.128	1.422	0.765	1.841	0.997
7	1.514	1.493	1.423	0.735	1.416	1.291
8	1.301	1.284	1.118	0.775	1.745	0.877
9	1.096	1.127	0.875	0.911	1.51	1.165
10	1.531	1.352	1.025	0.528	1.188	0.953
MEAN	1.5272	1.5101	1.4316	1.0022	1.6891	1.3104

SD	0.31342	0.31093	0.40022	0.42179	0.33362	0.46994
SE	0.09911	0.09833	0.12656	0.13338	0.1055	0.14861

SD7:Ten plants each of wild-type and MS1-2 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0, 150 and 200mM) of mannitol and corresponding value for each root length at 7 days.

SD8:

	Man					
	0mM		150mM		200mM	
Plant #	MS1-2	WT	MS1-2	WT	MS1-2	WT
1	2.987	2.297	2.512	1.194	2.151	3.36
2	2.381	2.793	1.968	1.261	2.791	2.575
3	2.199	2.587	1.608	1.413	1.701	1.433
4	2.316	2.258	1.698	2.09	1.981	1.35
5	2.108	2.278	2.066	1.367	3.16	1.285
6	2.2	2.177	1.85	1.561	1.977	2.081
7	2.275	2.472	2.344	1.236	2.208	1.39
8	2.234	2.302	1.971	1.437	1.903	1.198
9	2.241	2.252	2.17	1.449	2.231	1.549
10	2.409	2.552	2.601	1.789	1.788	1.702
MEAN	2.335	2.3968	2.0788	1.4797	2.1891	1.7923
SD	0.24584	0.19565	0.33033	0.27558	0.45677	0.69267
SE	0.07774	0.06187	0.10446	0.08715	0.14444	0.21904

SD8: Ten plants each of wild-type and MS1-2 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0, 150 and 200mM) of mannitol and corresponding value for each root length at 14 days.

SD9:

	Man					
	0mM		150mM		200mM	
Plant #	MS1-2-7	WT	MS1-2-7	WT	MS1-2-7	WT
1	1.025	1.821	1.898	1.693	1.573	1.487
2	2.133	2.176	2.03	1.554	1.781	1.138
3	2.407	2.001	1.788	1.651	1.942	1.248

4	2.151	2.166	1.948	1.586	1.606	1.332
5	1.02	1.012	2.046	1.617	1.964	1.258
6	1.951	1.982	1.371	1.184	1.075	1.585
7	1.835	1.755	1.228	1.323	1.199	1.31
8	1.807	1.771	1.039	1.919	0.985	1.369
9	1.942	1.642	1.263	1.206	1.956	1.442
10	1.835	1.87	1.372	1.362	1.564	1.356
MEAN	1.8106	1.8196	1.5983	1.5095	1.5645	1.3525
SD	0.45411	0.33319	0.38008	0.23461	0.36713	0.12846
SE	0.1436	0.10536	0.12019	0.07419	0.1161	0.04062

SD9: Ten plants each of wild-type and MS1-2-7 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0, 150 and 200mM) of mannitol and corresponding value for each root length at 7 days.

SD10:

	Man					
	0mM		150mM		200mM	
Plant #	MS1-2-7	WT	MS1-2-7	WT	MS1-2-7	WT
1	2.447	2.497	2.687	1.921	2.18	1.213
2	2.239	1.913	2.275	1.601	2.499	1.212
3	2.228	2.396	2.321	2.233	2.712	1.756
4	2.433	2.18	2.457	2.073	2.601	1.478
5	2.116	2.036	2.101	2.104	2.712	1.574
6	2.037	2.337	2.001	2.225	2.841	1.598
7	2.167	2.003	2.722	2.274	2.387	1.682
8	2.238	1.901	2.389	2.227	2.444	1.863
9	2.492	2.209	2.519	2.294	2.582	1.722
10	2.129	2.584	2.154	1.779	2.045	1.844
MEAN	2.2526	2.2056	2.3626	2.0731	2.5003	1.5942
SD	0.15506	0.24278	0.2405	0.23451	0.24685	0.23338
SE	0.04903	0.07677	0.07605	0.07416	0.07806	0.0738

SD10: Ten plants each of wild-type and MS1-2-7 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0, 150 and 200mM) of mannitol and corresponding value for each root length at 14 days.

SD11:

	Man					
	0mM		150mM		200mM	
Plant #	MS1-2-14	WT	MS1-2-14	WT	MS1-2-14	WT
1	2.229	1.803	1.612	1.542	1.252	0.612
2	1.513	2.053	1.402	1.351	1.075	0.586
3	1.629	2.031	1.178	1.69	0.837	0.728
4	1.4	1.542	1.52	1.305	1.181	0.91
5	1.703	1.288	1.538	1.773	0.806	0.448
6	1.241	1.451	1.124	1.278	0.733	0.665
7	1.331	1.218	1.173	1.137	0.876	0.597
8	1.047	1.056	1.322	0.94	1.128	0.479
9	2.125	1.913	1.281	1.312	0.445	0.697
10	1.688	1.36	1.156	1.039	1.086	0.399
MEAN	1.5906	1.5715	1.3306	1.3367	0.9419	0.6121
SD	0.37181	0.35659	0.17857	0.26843	0.24739	0.15024
SE	0.11758	0.11276	0.05647	0.08488	0.07823	0.04751

SD11:Ten plants each of wild-type and MS1-2-14 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0, 150 and 200mM) of mannitol and corresponding value for each root length at 7 days.

SD12:

	Man					
	0mM		150mM		200mM	
Plant #	MS1-2-14	WT	MS1-2-14	WT	MS1-2-14	WT
1	2.027	2.387	1.71	2.325	1.302	0.95
2	1.994	1.812	1.685	2.066	1.932	1.422
3	2.095	2.267	2.117	1.469	0.884	1.313
4	2.136	2.137	2.279	1.587	1.005	1.258

5	2.759	1.955	2.688	1.94	1.344	1.088
6	1.706	2.095	2.079	2.016	1.281	0.862
7	1.686	2.178	2.406	1.573	1.013	0.889
8	2.189	1.909	2.794	1.334	1.476	0.912
9	2.087	2.016	2.527	2.102	1.19	1.322
10	2.15	1.901	1.769	0.981	1.017	1.4
MEAN	2.0829	2.0657	2.2054	1.7393	1.2444	1.1416
SD	0.29561	0.18046	0.40262	0.41652	0.30485	0.22488
SE	0.09348	0.05707	0.12732	0.13171	0.0964	0.07111

SD12: Ten plants each of wild-type and MS1-2-14 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0, 150 and 200mM) of mannitol and corresponding value for each root length at 14 days.

DROUGHT STRESS

SD13:

	PEG					
	0%		3%		5%	
Plant #	MS1-2	WT	MS1-2	WT	MS1-2	WT
1	1.551	1.528	2.298	0.874	1.381	0.934
2	1.238	1.657	1.729	0.872	1.219	1.134
3	1.389	1.244	2.415	0.946	1.51	0.765
4	1.165	1.296	1.586	0.991	1.234	1.042
5	1.468	1.321	2.574	1.408	1.521	0.655
6	1.101	0.776	2.107	1.201	1.467	0.942
7	1.043	1.02	1.269	0.83	1.338	0.7
8	0.988	0.81	1.357	1.658	0.849	0.836
9	1.148	1.369	1.305	0.93	1.036	0.929
10	1.054	1.126	1.132	1.116	1.297	1.153
MEAN	1.2145	1.2147	1.7772	1.0826	1.2852	0.909
SD	0.19285	0.28643	0.53069	0.26959	0.21361	0.17134
SE	0.06099	0.09058	0.16782	0.08525	0.06755	0.05418

SD13: Ten plants each of wild-type and MS1-2 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations of (0,3 and 5%) PEG and corresponding value for each root length at 7 days.

SD14:

	PEG					
	0%		3%		5%	
Plant #	MS1-2	WT	MS1-2	WT	MS1-2	WT
1	1.834	1.795	3.124	1.961	1.829	1.907
2	2.217	1.783	2.514	1.582	2.713	1.664
3	1.902	1.876	3.2	1.696	1.853	0.344
4	1.725	1.636	3.013	1.573	1.644	2.103
5	1.881	1.508	1.769	1.884	2.882	1.696
6	2.033	1.61	1.766	2.108	2.234	0.684
7	1.588	1.758	1.651	1.433	2.507	1.364
8	2.247	1.937	2.187	2.069	1.769	1.091
9	2.03	2.168	1.943	1.619	1.612	0.715
10	1.216	1.961	2.043	1.586	3.006	1.276
MEAN	1.8673	1.8032	2.321	1.7511	2.2049	1.2844
SD	0.30647	0.19325	0.59938	0.23556	0.53456	0.57582
SE	0.09692	0.06111	0.18954	0.07449	0.16904	0.18209

SD14: Ten plants each of wild-type and MS1-2 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations of (0,3 and 5%) PEG and corresponding value for each root length at 14 days.

SD15:

	PEG					
	0%		3%		5%	
Plant #	MS1-2-7	WT	MS1-2-7	WT	MS1-2-7	WT
1	1.902	1.921	1.347	1.359	2.224	1.145
2	2.024	1.615	0.647	0.819	2.329	1.038
3	1.725	1.919	1.421	2.079	2.041	1.698
4	1.767	2.031	1.773	1.058	1.672	1.496
5	1.804	1.774	1.325	1.347	2.129	1.668

6	1.87	1.921	2.108	1.473	2.116	1.317
7	1.816	1.884	0.974	0.868	1.473	1.644
8	1.955	1.707	0.924	0.678	1.254	1.29
9	1.923	1.963	1.998	1.54	1.09	1.593
10	1.842	1.869	1.034	1.21	1.433	1.357
MEAN	1.8628	1.8604	1.3551	1.2431	1.7761	1.4246
SD	0.09064	0.12572	0.48255	0.41355	0.44476	0.23033
SE	0.02866	0.03976	0.1526	0.13078	0.14065	0.07284

SD15:Ten plants each of wild-type and MS1-2-7 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations of (0,3 and 5%) PEG and corresponding value for each root length at 7 days.

SD16:

	PEG					
	0%		3%		5%	
Plant #	MS1-2-7	WT	MS1-2-7	WT	MS1-2-7	WT
1	3.222	3.095	2.315	1.948	1.674	2.626
2	3.538	2.898	2.527	1.579	3.148	2.107
3	2.617	2.76	2.253	1.663	2.439	2.815
4	2.705	2.807	2.155	1.81	2.008	3.42
5	2.583	3.02	2.296	1.987	2.211	1.457
6	3.2	2.864	2.148	1.844	2.658	2.45
7	3.205	2.734	2.251	1.703	2.162	3.132
8	3.247	2.711	1.361	1.42	2.717	2.026
9	3.098	2.849	2.05	2.172	3.361	1.363
10	2.236	3.006	1.829	1.232	1.401	1.513
MEAN	2.9651	2.8744	2.1185	1.7358	2.3779	2.2909
SD	0.40421	0.13019	0.32269	0.27891	0.61624	0.71958
SE	0.12782	0.04117	0.10204	0.0882	0.19487	0.22755

SD16: Ten plants each of wild-type and MS1-2-7 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations of (0,3 and 5%) PEG and corresponding value for each root length at 14 days.

SD17:

	PEG					
	0%		3%		5%	
Plant #	MS1-2-14	WT	MS1-2-14	WT	MS1-2-14	WT
1	1.712	1.74	1.862	1.291	1.251	0.369
2	1.662	1.885	2.107	1.028	2.037	0.747
3	1.778	1.779	1.242	1.209	0.926	1.248
4	1.605	1.6	3.16	1.144	2.668	0.486
5	1.532	1.618	1.737	0.974	1.527	1.508
6	1.699	1.752	0.612	1.353	1.185	1.506
7	1.542	1.562	2.369	1.067	1.597	0.701
8	1.876	1.903	1.225	1.198	1.228	1.117
9	1.957	1.733	1.153	1.116	1.488	1.117
10	2.083	1.859	1.47	1.294	1.83	1.067
MEAN	1.7446	1.7431	1.6937	1.1674	1.5737	0.9866
SD	0.18065	0.11997	0.72597	0.12406	0.50356	0.39741
SE	0.05713	0.03794	0.22957	0.03923	0.15924	0.12567

SD17: Ten plants each of wild-type and MS1-2-14 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0,3 and 5%) of PEG and corresponding value for each root length at 7 days.

SD18:

	PEG					
	0%		3%		5%	
Plant #	MS1-2-14	WT	MS1-2-14	WT	MS1-2-14	WT
1	2.451	2.339	2.129	2.625	2.209	1.23
2	2.252	2.464	3.295	2.319	2.684	1.176
3	2.695	2.119	2.754	2.168	1.995	2.075
4	2.27	1.979	2.698	2.11	2.266	1.464
5	2.031	2.327	3.093	1.899	1.889	1.953
6	2.27	2.286	2.785	2.787	1.763	1.738
7	2.142	2.374	2.946	2.002	2.436	1.695

8	2.632	2.755	2.801	2.504	2.216	1.766
9	2.318	2.545	2.671	2.364	2.293	1.725
10	2.456	2.46	2.602	2.138	2.35	1.883
MEAN	2.3517	2.3648	2.7774	2.2916	2.2101	1.6705
SD	0.20774	0.21635	0.31018	0.28236	0.26967	0.29572
SE	0.06569	0.06842	0.09809	0.08929	0.08528	0.09351

SD18: Ten plants each of wild-type and MS1-2-14 sip428-silenced lines were used for root length data collection. Data show root length of plants in different concentrations (0,3 and 5%) of PEG and corresponding value for each root length at 14 days.

VITA

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