Overexpression of a miR393-Resistant Form of Transport Inhibitor Response Protein 1 (mTIR1) Enhances Salt Tolerance by Increased Osmoregulation and Na\textsuperscript{+} Exclusion in Arabidopsis thaliana

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Soil salinity is a common environmental stress factor that limits agricultural production worldwide. Plants have evolved different strategies to achieve salt tolerance. miR393 has been identified as closely related to biotic and abiotic stresses, and targets F-box genes that encode auxin receptors. The miR393–TIR1/AFB2/AFB3 regulatory module was discovered to have multiple functions that manipulate the auxin response. This study focused on miR393 and one of its targets, TIR1, and found that they played potential roles in response to salt stress. Our results showed that overexpression of a miR393-resistant TIR1 gene (mTIR1) in Arabidopsis clearly enhanced salt stress tolerance, which led to a higher germination rate, less water loss, reduced inhibition of root elongation, delayed senescence, decreased death rate and stabilized Chl content. These plants accumulated more proline and anthocyanin, and displayed enhanced osmotic stress tolerance. The expression of some salt stress-related genes was altered, and sodium content can be reduced in these plants under salt stress. We proposed that highly increased auxin signaling by overexpression of mTIR1 may trigger auxin-mediated downstream pathways to enhance plant salt stress resistance by osmoregulation and increased Na\textsuperscript{+} exclusion.

Keywords: Arabidopsis thaliana • MiR393–TIR1 • Osmoregulation • Salinity • Tolerance.

Abbreviations: ACS, 1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE; ACT2, ACTIN2; AFB, AUXIN SIGNALING F BOX PROTEIN; ALDH10A8, ALDEHYDE DEHYDROGENASE 10A8; ALDH10A9, ALDEHYDE DEHYDROGENASE 10A9; ANOVA, analysis of variance; AVP1, ARABIDOPSIS THALIANA V-PPASE1; CMO, CHOLINE MONOOXYGENASE; GB, glycine betaine; GH3, GRETCHEN HAGEN3; GUS, β-glucuronidase; miR, microRNA; HX1, NA\textsuperscript{+}/H\textsuperscript{+} EXCHANGER1; qRT-PCR, quantitative real-time PCR; SOS1, SALT OVERLY SENSITIVE1; TIR1, TRANSPORT INHIBITOR RESPONSE PROTEIN1; UBQ5, UBQUITIN5.

Introduction

Over 800 Mha of land, which accounts for >6% of the world’s total land area, are affected by salt (Munns and Tester 2008). Sodium chloride is the most soluble, abundant and widespread salt released from the weathering of rocks (Szabolcs 1989, Rengasamy 2010). Soil salinity stresses plants by disrupting homeostasis in water potential and ion distribution (Zhu 2001, Borsani et al. 2003). It is harder for roots to take up water under high concentrations of salt in soil, and accumulating high concentrations of salt ions can be toxic for plants (Hasegawa et al. 2000). Disrupting ion and water homeostasis leads to molecular damage, growth arrest and even death. Plants use three interconnected strategies to achieve salt tolerance. First, damage must be prevented or alleviated. Secondly, homeostatic conditions must be re-established in the new, stressful environment. Thirdly, growth must resume, albeit at a reduced rate (Zhu 2001). It is not surprising that all plants have evolved mechanisms to regulate salt accumulation. It is also not surprising that plants have evolved mechanisms to tolerate the low soil water potential caused by salinity (Zhu 2002, Munns and Tester 2008).

MicroRNAs (miRNAs) have been shown to play important regulatory roles by targeting complementary mRNA transcripts for cleavage or translational repression in plants (Carrington and Ambros 2003, Voinnet 2009). Plant miRNAs have been proven to play crucial roles in plant growth and development (Jones-Rhoades et al. 2006, Huijser and Schmid 2011). They also have indirect regulatory functions in production of trans-acting small interfering RNA (ta-siRNA) (Vazquez et al. 2004, Yoon et al. 2010) and modification of signal transduction systems (Yang et al. 2006, Meng et al. 2010). Furthermore, many plant miRNAs are also involved in stress responses against various environmental factors (Sunkar and Zhu 2004, Liu et al. 2008, Covarrubias and Reyes 2010, Ding et al. 2013).
One of the conserved miRNA families in plants, the MIR393 genes, have been found in different plant species (Navarro et al. 2006). In Arabidopsis, the targets of miR393 are F-box genes that encode auxin receptors [TRANSPORT INHIBITOR RESPONSE PROTEIN1 (TIR1), AUXIN SIGNALING F BOX PROTEIN 2 (AFB2) and AFB3] (Jones-Rhoades and Bartel 2004, Kepinski and Leyser 2005, Dharmasiri et al. 2005a, Dharmasiri et al. 2005b). The miR393–TIR1/AFB2/AFB3 regulatory module has been discovered to have multiple functions that manipulate the auxin responses (Windels et al. 2014), such as controlling the root architecture (Vidal et al. 2010), regulating leaf development (Si-Ammour et al. 2011) and maintenance of normal plant growth (Chen et al. 2011). This miRNA has also been identified to be closely related to biotic and abiotic stresses (Sunkar and Zhu 2004, Navarro et al. 2006). Similar reports from research in rice prove that the biological functions of miR393 and its regulation mechanism via the auxin pathway are also conserved in higher plants (Gao et al. 2011, Bian et al. 2012, Xia et al. 2012).

Research showed that members of the miR393 family participate in salt stress responses, but whether the interaction between miR393 and its target genes affects plant salt tolerance remains unknown. In our previous work, we generated a miR393-resistant TIR1 gene (mTIR1) and found that 35S::mTIR1 plants displayed many developmental abnormalities (Chen et al. 2011). In the study herein, we focused on the expression of miR393 and TIR1 under salt stress, the relationship between salt responses and the miR393–TIR1 interaction system, and the possible mechanism of salt tolerance, in order to illustrate the potential roles of miR393 and TIR1 in salt response.

Results

miR393 and its target TIR1 are involved in salt stress responses

In order to better understand whether the miR393–TIR1 regulatory module is involved in responsiveness to salt stress in plant, we measured the precursor abundance of MIR393a and MIR393b and the expression level of TIR1 under salt treatment. Our data showed that both the transcripts of pri-393a and TIR1 from 10-day-old wild-type seedlings were increased more obviously after 6h of treatment with 200 mM NaCl than those of pri-393b (Fig. 1A). We also used pMIR393a::GUS, pMIR393b::GUS and pTIR1::GUS transgenic lines from our previous work (Chen et al. 2011) to verify this result. After the same NaCl treatment, pMIR393a::GUS transgenic seedlings showed a more obvious increase of β-glucuronidase (GUS) intensity in the mesophyll and hypocotyls than pMIR393b::GUS seedlings (Fig. 1B). The pTIR1::GUS plants were too deeply stained to distinguish, so we used quantitative real-time PCR (qRT-PCR) to analyze GUS gene expression in detail. The results showed that GUS genes were promoted significantly in both pMIR393a::GUS and pTIR1::GUS transgenic lines (Fig. 1C). These data indicated that miR393 and its target TIR1 are involved in salt stress responses and both miR393 and its target TIR1 were up-regulated after NaCl treatment.

Salt stress tolerance is enhanced in mTIR1-overexpressing plants

To investigate the role of the miR393–TIR1 module under salt conditions, we used a tir1-1 mutant and transgenic Arabidopsis
plants harboring constructs of 35S::MIR393 and 35S::mTIR1 (a miR393-resistant form of TIR1) from our previous work (Chen et al. 2011).

The germination rate under salt stress was first determined. We found that all seedlings were nearly 100% germinated after 2 d of growth on control B5 medium (Fig. 2A). Under growth conditions with an extra 150 mM NaCl, the germination rate of 35S::mTIR1 lines reached 90% after 2 d and 100% within 4 d; however, the wild type needed >5 d to reach a germination rate of only 90% (Fig. 2B).

Root growth can be inhibited under exogenous NaCl, and the inhibition can be enhanced with an increase in NaCl concentration. The primary roots of 35S::mTIR1 plants were significantly shorter than those of the wild type under normal conditions, as previously described. However, the differences in root length between 35S::mTIR1 and the wild type were narrowed under 100 mM NaCl and even eliminated under higher salt media (Fig. 2C). In our experiment, similar lengths of roots were observed in all seedlings under 150 mM NaCl, with the root elongation rate reduced by almost 80% in the wild type, but by ~65% in 35S::mTIR1, which suggested reduced inhibition of root elongation in mTIR1-overexpressing plants (Fig. 2C). The fresh weight accumulation of these lines was then measured. While plant growth in the control was similar, 35S::mTIR1 plants grew to a larger size and gained more fresh weight than the wild type on both high salt media (Fig. 2D).

These results demonstrated that overexpression of mTIR1 led to reduced saline sensitivity and probably enhanced plant salt stress tolerance. However, no changes of salt sensitivity or tolerance were found in 35S::MIR393 lines (data not shown).

Overexpression of mTIR1 leads to longevity and delayed senescence under salt stress

We further tested the growth status and survival rate of wild type Col-0, 35S::mTIR1-7, 35S::mTIR1-9, 35S::MIR393a-6, 35S::MIR393b-1 and mutant tir1-1 under salt stress. Five-day-old seedlings were transferred to B5 medium with 300 mM NaCl for growth analysis. We divided the growth status into three categories distinguished by cotyledon color: green as normal, yellowish green as subhealthy, and white as dead. The percentage of normal seedlings was decreased after 2 d of growth on extra high NaCl in all lines, with cotyledons turning yellowish, but in 35S::mTIR1 plants the percentages of normal seedlings were still >60% (Fig. 3A). Dead plants with totally white cotyledons appeared in wild-type and 35S::MIR393 plants after 4 d of growth, with the percentages of normal cotyledons dropping to <10%. However, the percentage remained about 40% in 35S::mTIR1 plants and no dead plants were found (Fig. 3B). Six days growth on extra high NaCl was fatal to most of the plants, but there were still 10–20% of subhealthy yellowish 35S::mTIR1 seedlings that had survived (Fig. 3C).

We then watered 3-week-old plants growing in soil with or without extra high NaCl for another week to analyze the change...
of growth status and Chl content. All plants flowered normally and stayed green in the control (Fig. 4A). However, the green color of rosette leaves faded in all wild-type and TIR1-inhibited plants (35S::MIR393 and tir1-1) after the salt treatment, with visible white spots appearing simultaneously. In contrast, leaves from both of the two 35S::mTIR1 lines stayed green and the color even intensified (Fig. 4B). We collected rosette leaves from these lines to measure their relative Chl content. The level of accumulation of Chl was less than half in all wild-type and TIR1-inhibited plants under high salt; however, the Chl level in 35S::mTIR1 plants was stable (Fig. 4C). These data illustrated that overexpression of mTIR1 leads to delayed senescence and longevity, with a somewhat decreased death rate, and probably enhanced salt tolerance by maintaining the stability of Chl metabolism.

**Exogenous NaCl highly promotes proline and anthocyanin accumulation in mTIR1-overexpressing plants**

High salinity often leads to osmotic stress and triggers the alteration of proline content, overaccumulation of which could be an indicator of enhanced stress tolerance (Szabados and Savoure 2010). We used the same plants as used for Chl content analysis for assessment of proline abundance. Proline levels were increased sharply in all plants after salt treatment (Fig. 5A). Compared with wild-type plants, proline levels were much higher in 35S::mTIR1 plants and even reached 700–800 μg g⁻¹ FW in saline conditions. In comparison, 35S::MIR393 and tir1-1 plants had a slightly lower proline content than wild-type plants under salt treatment (Fig. 5A).

Our photographs showed that the color of 35S::mTIR1 plant leaves became darker under salt treatment (Fig. 4B), which indicated an enhanced pigmentation. Anthocyanin plays an essential role in regulating osmotic equilibrium under multiple stresses (Gould 2004, Koes et al. 2005, Lepiniec et al. 2006); therefore, we determined the accumulation of anthocyanin in the same tissues collected for Chl analysis. The amount of anthocyanin was undetectable under normal conditions in all lines, but showed up clearly under extra high NaCl treatment. Interestingly, the levels of anthocyanin increased greatly in mTIR1-overexpressing plants and was >10-fold that of the wild type (Fig. 5B). These data proved that proline and anthocyanin accumulated more in mTIR1-overexpressing plants.

**Overexpression of mTIR1 leads to osmotic stress tolerance**

Seedling growth was also detected in wild type Col-0, 35S::mTIR1-7, 35S::mTIR1-9, 35S::MIR393a-6, 35S::MIR393b-1 and mutant tir1-1 under osmotic stress. Five-day-old seedlings were transferred to B5 medium with 200 mM mannitol for growth analysis. After a 9 d treatment, root growth was clearly inhibited under osmotic stress in wild-type, miR393-overexpressing plants and mutant tir1-1, with shorter length and fewer lateral roots, but not in 35S::mTIR1 plants (Fig. 6A). Compared with control plants, only 35S::mTIR1 plants showed an elongated primary root with longer root length under mannitol treatment (Fig. 6B). Fresh weight accumulation was then calculated. Although fresh weight accumulation of 35S::mTIR1 plants was slightly lower than that in controls, they gained more fresh weight than the others on high osmotic
stress media (Fig. 6C). These results demonstrated that overexpression of mTIR1 probably led to enhanced plant osmotic stress tolerance.

Expression analysis of salt stress-related genes

To elucidate the higher salt tolerance of mTIR1-overexpressing plants, we determined the expression of several NaCl-induced genes that are involved in plant salt stress tolerance by RT-PCR. Glycine betaine (GB) provides tolerance to cells under osmotic stress by stabilizing the structure of proteins and by adjusting the osmotic potential in the cytoplasm (Bartels and Sunkar 2005, Kotchoni et al. 2006, Ashraf and Foolad 2007). In plants, GB is synthesized through a two-step oxidation of choline via betaine aldehyde (Takabe et al. 2006). Two enzymes, choline monooxygenase (CMO) and NAD-dependent betaine aldehyde dehydrogenase (BADH), are involved (Rathinasabapathi et al. 1997, Missihoun et al. 2011). We compared the relative expression levels of Arabidopsis CMO (At4g29890), ALDH10A8 (At1g74920) and ALDH10A9 (At3g68170) genes in 35S:mTIR1 plants and the wild type. Transcripts of these genes were slightly increased <1.5-fold under salinity in the wild type (Fig. 7A–C). However, a further increase of ALDH10A8 and ALDH10A9, but not of CMO, was observed in 35S:mTIR1, leading to a nearly doubled relative level (Fig. 7B, C).

In Arabidopsis, Na⁺ can be exported out of the cell by the Na⁺/H⁺ antiporter SALT OVERLY SENSITIVE1 (SOS1) located on the plasma membrane (Shi et al. 2000), and can also be transported into the vacuole by another Na⁺/H⁺ antiporter, NHX1, located on the tonoplast membrane with the help of a H⁺-pump pyrophosphatase ARABIDOPSIS THALIANA V-PPASE1 (AVP1; Apse et al. 1999, Gaxiola et al. 2001). We also compared the relative expression levels of Arabidopsis SOS1 (At1g01980), NHX1 (At5g27150) and AVP1 (At1g15690) genes in 35S:mTIR1 plants and the wild type under salinity. Our data showed that the increase in SOS1 in 35S:mTIR1 plants was more obvious than in the wild type Col-0 under salt treatment, and about 2.5-fold that in the control (Fig. 7D). No notable change of AVP1 was seen, but there was a 3-fold up-regulation of NHX1 in all lines under salinity, with no differences between 35S:mTIR1 plants and the wild type (Fig. 7E, F), indicating that the two genes were not affected by an overaccumulation of mTIR1.

Fig. 4 Overexpression of mTIR1 stabilized the Chl content under salt stress. Three-week-old plants growing in soil were watered with or without extra NaCl for another week to analyze the change of growth status and Chl content. Plants watered without (A) or with (B) 300 mM NaCl for another week were photographed. Different plant lines were distinguished and arranged as in the diagram in the top right. (C) Relative content of Chl (μg g⁻¹ FW) in the wild type, 35S:mTIR1, 35S:MIR393 and the tir1-1 mutant. Error bars represent the SD. Different letters represent significantly different values at P < 0.05 (Duncan’s multiple range test).
Discussion

The miR393–TIR1 regulatory module play roles in salt stress response

Many plant miRNAs have been reported to participate in stress responses against different environmental factors (Sunkar et al. 2007, Covarrubias and Reyes 2010, Ding et al. 2013). Mature miR393 has also been shown to be a conserved miRNA among different plant species such as Arabidopsis, rice, maize, poplar, *Medicago* and *Brassica napus* (Jones-Rhoades and Bartel 2004, Tuskan et al. 2006, Jagadeeswaran et al. 2009, Zhang et al. 2009, Bian et al. 2012). Previous studies have also shown that miR393 can be induced by different stresses including salt (Sunkar and Zhu 2004, Liu et al. 2008). In this work, we show that miR393 and its target gene TIR1 can be induced by NaCl in Arabidopsis seedlings. Analysis of precursor transcripts and promoter::GUS reporter lines confirmed that pri-393a was mainly induced in response to NaCl treatment, which might illustrate a locus-specific control of MIR393 transcription under salinity. Salinity treatment increases the level of both pri-393a and TIR1 (Fig. 1), inferring a co-ordinate expression pattern between miR393 and TIR1 under salt conditions. To look into this, we determined the expression level of MIR393 in the wild type, tir1-1, the tir1-1/afb1-1/afb2-1/afb3-1 quadruple mutant and 35S::mTIR1 under both control and salinity conditions. The data showed that the expression of pri-393a was notably elevated in both Col-0 and 35S::mTIR1 lines under salt treatment, and was higher in TIR1-overaccumulating lines. However, the deficient TIR1 functions in tir1-1 and tir1-1/afb1-1/afb2-1/afb3-1 led to a relatively stable pri-393a level under salinity (Fig. 9A). No similar patterns were found in pri-393b, with the levels unchanged in all these lines under salt (Fig. 9B). It is possible that, under salinity conditions, the increased TIR1 level might regulate the level of MIR393a in Arabidopsis in a feedback mechanism.

We found that expression of the promoter-driven GUS could not produce a dramatic increase of TIR1 (Fig. 1). This is probably due to the efficiency of the promoter. It is also possible that other unidentified mechanisms might also contribute to the complex regulation under salinity. Plant miRNAs and their targets orchestrate prototypical regulatory circuits at the systems level, including spatial restriction, temporal regulation, mutual exclusion of the miRNA and target gene, as well as the dampening of target gene expression (Voinnet 2009). We propose that the miR393–TIR1 regulatory module plays a role in salt stress response, which could influence the type of circuit involved.

Overexpression of mTIR1 enhances the salt stress tolerance

High concentrations of salts in the soil make it harder for roots to extract water, and high concentrations of salts within the plant can be toxic. Salt tolerance mechanisms in plants have been discussed in various ways and adequately reviewed (Zhu 2002, Munns and Tester 2008). In this work, we reported that our previously obtained transgenic Arabidopsis could

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**Sodium content can be reduced in 35S::mTIR1 transgenic plants under salt stress**

To gain insight into whether the sodium content could be affected by overexpression of mTIR1, we measured the relative Na\(^+\) content of Col-0 and 35S::mTIR1 plants under salt stress. Ten-day-old plants were transferred onto B5-agar medium with or without added 200 mM NaCl for 6 h. Our results showed that the Na\(^+\) content was lower in 35S::mTIR1 plants than in the wild type under control conditions, and was elevated significantly in all plants under salt treatment (Fig. 8A). However, the relative Na\(^+\) content reached about 8 mg \(g^{-1}\) DW in 35S::mTIR1 plants, which increased no more than 1.4-fold under salt, while it reached 16 mg \(g^{-1}\) DW in the wild type with a 2.5-fold change in the control at the same time. These results clearly showed that the sodium content can be reduced in 35S::mTIR1 transgenic plants under salt stress.
Fig. 6 Enhanced osmotic stress tolerance in mTIR1-overexpressing plants. Wild-type Col-0, 35S::mTIR1, 35S::MIR393 and mutant tir1-1 seedlings were tested for osmotic stress tolerance. (A) Five-day-old seedlings transferred onto new culture medium supplemented by 0 or 200 mM mannitol for another 9 d were photographed. Primary root length (B) and fresh weight (FW) accumulation (C) were measured. Error bars represent the SD. Asterisks denote a significant difference from the control plants (P < 0.05, t-test) and different letters represent significantly different values at P < 0.05 (Duncan’s multiple range test).

Fig. 7 Expression analysis of salt stress-related genes. qRT-PCR analysis of (A) Arabidopsis CMO/At4g29890, (B) ALDH10A8/At1g74920, (C) ALDH10A9/At3g48170, (D) SOS1/At2g01980, (E) AVP1/At1g15690 and (F) NHX1/At5g27750 in 35S::mTIR1 plants and Col-0 in response to exogenous NaCl. Ten-day-old seedlings were transferred onto the same B5 medium with or without 200 mM NaCl for 6 h. Quantifications were normalized to the expression of UBQ5. The relative expression levels in control Col-0 plants were set to 1.0. Error bars represent the SD from three independent experiments.
enhance salt tolerance and delay senescence, probably regulated by the miR393–TIR1 interaction. **35S::mTIR1** transgenic plants overexpress a miRNA-resistant version of TIR1, in which six silent mutations have been introduced into the miR393 complementary site that disrupts miRNA targeting without altering the corresponding amino acid sequences (Chen et al. 2011). These lines had dramatically increased TIR1 mRNA levels and could mimic a **mir393** loss-of-function mutant. Our results showed that overexpression of the **mTIR1** gene clearly enhanced salt stress tolerance, which led to a higher germination rate, less water loss, reduced inhibition of root elongation, delayed senescence, a decreased death rate and stabilized Chl content (Figs. 2–4). In contrast, we supposed that both **35S::MIR393** transgenic plants and the **tir1-1** mutant should have the opposite characteristics to **35S::mTIR1** plants of being salt oversensitive because of their function-retarded TIR1 gene. However, there were no apparent alterations observed in these lines compared with wild type Col-0 plants. A reasonable explanation is that one of the TIR1 homologous genes, AFB1, is partially resistant to miR393 regulation, but functions in a redundant way (Navarro et al. 2006). We had also planned to check the growth status of the **tir1-1/afb1-1/afb2-1/afb3-1** quadruple mutant under salt stress. However, an array of phenotypes is observed in this mutant that can be divided into three major classes under normal conditions (Dharmasiri et al. 2005b). The inconsistency of the phenotype made this quadruple mutant unsuitable for analysis of its growth status.

**Possible mechanism of the miR393–TIR1 regulatory module in salt response and tolerance**

The miR393–TIR1 interaction has multiple regulatory functions including regulation of development and stress response (Sunkar and Zhu 2004, Parry et al. 2009, Si-Ammour et al. 2011, Windels et al. 2014). In this study, we showed that this interaction is involved in the salt stress response. TIR1 protein functions as an auxin receptor involved in auxin perception, signaling and response (Ruegger et al. 1998, Kepinski and Leyser 2005, Dharmasiri et al. 2005a). Then what is the role of auxin in the **mTIR1** overexpression-mediated salt stress tolerance? Salt stress has been reported to stimulate the production of ethylene (Wang et al. 2002), and ethylene stimulates auxin biosynthesis and basipetal auxin transport (Růžička et al. 2007). Auxin biosynthesis, transport, signaling and response are required for the ethylene-induced growth inhibition in roots (Stepanova et al. 2007). Plant growth regulation is related to auxin homeostasis in the stress adaptation response (Park et al. 2007).
The distribution and localization of auxin is correlated with root system architecture under salt stress (Sun et al. 2008, Wang et al. 2009, Zhao et al. 2011). These reports inferred that auxin signaling could be enhanced by exogenous salt stress and inhibited normal plant growth in some particular tissues, which might be an adaptation to stress response. In our previous microarray data, two IAA amido synthetase genes (At2g23170/GH3.3 and At4g27260/GH3.5) and three 1-amino-cyclopropane-1-carboxylate synthase genes (At2g22810/ACS5, At4g26200/ACS7 and At4g08040/ACS11) were up-regulated significantly in mTIR1-overexpressing plants (Chen et al. 2011). qRT-PCR analysis showed a >10-fold elevation of GH3.3 and a >3-fold up-regulation of the other genes in 35S::mTIR1 plants (Supplementary Fig. S1). Considering that the up-regulated ethylene and IAA synthase gene are very likely to enhance further the auxin accumulation in 35S::mTIR1 plants, combined with the similar pleiotropic phenotypes of IAA-overproduced plants described in our previous work, we believe that the auxin signaling pathway has been enhanced greatly in 35S::mTIR1 lines and played important roles in salt stress response.

The mechanisms of salinity tolerance fall into three categories: tolerance to osmotic stress, Na⁺ exclusion from leaf blades or Na⁺ compartmentalization at the cellular and intracellular level to avoid toxic concentrations (Munns and Tester 2008). We discovered that overexpression of mTIR1 could lead to overaccumulation of osmoregulation substances such as proline and anthocyanin. Further experiments also proved that 35S::mTIR1 plants were more tolerant to osmotic stress than the wild type (Figs. 5, 6). Genes encoding GB synthesis enzymes were highly up-regulated in 35S::mTIR1 under stress, which provided indirect evidence that GB osmoregulation substance might also play a role in this module. High concentrations of Na⁺ can be toxic to plant cells, but plants have evolved a system of Na⁺ exclusion or compartmentalization to cope with this. SOS1 encodes a putative Na⁺/H⁺ antiporter on the plasma membrane, and is responsible for Na⁺ exclusion in plants (Shi et al. 2000, Quintero et al. 2002). In the present study, the SOS1 level was increased about 2.5-fold in 35S::mTIR1 plants compared with the wild-type Col-0 under salt treatment, suggesting that overexpression of mTIR1 could export more Na⁺ out of the cell and enhance salt tolerance. Determination of the relative Na⁺ content of the plant also confirmed that the sodium content can be reduced in 35S::mTIR1 transgenic plants under salt stress (Figs. 7, 8).

Herein, we proposed that increased auxin signaling by overexpression of mTIR1 might trigger many auxin-mediated downstream pathways to enhance plant salt stress resistance by increased osmoregulation and Na⁺ exclusion.

Materials and Methods

Plant materials and growth conditions

The Arabidopsis thaliana tir1-1 mutant were kindly provided by Mark Estelle (Section of Cell and Developmental Biology, The University of California, San Diego). All the other lines were obtained in our previous work (Chen et al. 2011). Wild-type (Col-0), mutant and transgenic plant seeds were surface sterilized with 70% ethanol and 10% bleach. Sterilized seeds were sown on B5-agar plates. Plates were vernalized in darkness for 2 d at 4°C and then transferred to a growth chamber at 22°C and 70% humidity under a 16 h light/8 h dark photoperiod.

Gene expression analysis

Expression levels of pri-393a, pri-393b and TIR1 in 2-week-old wild-type, 35S::mTIR1, 35S::miR393 and tir1-1 seedlings were verified by semi-quantitative RT-PCR using ACT2 as a control. Thirty-two cycles for pri-393a and 39-39, 27 cycles for TIR1 and 22 cycles for ACT2 were used for amplifications. Primers of pri-MIR393 were designed from the precursor stem–loop, and primers of TIR1 were designed from each side of the miR393 cleavage site.

For NaCl treatment, 10-day-old seedlings were transferred onto the same medium with or without 200 mM NaCl for 6 h. Then total RNA was isolated using TRIzol reagent (Invitrogen), and treated with RNase-free DNase I (TAKARA). Treated RNA (1 μg) was used for the first-strand cDNA synthesis using a PrimeScript RT Reagent Kit (TAKARA). Real-time PCR was performed using Mastercycler ep realplex2 (Eppendorf) with the SYBR Premix Ex Taq (Perfect Real Time) Kit (TAKARA). Relative transcript levels were all normalized using UBQ5 as a standard. All primers used in this work are listed in Supplementary Table S1. In all experiments, at least 40 seedlings were used per Petri dish and all experiments were repeated three times.

Histochemical detection of GUS activity

Histochemical localization of GUS staining was performed by incubating seedlings in a solution of 1 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 0.5 mM potassium ferricyanide, 0.5 mM potassium hexacyanoferrate, 0.1% Triton X-100, 50 mM sodium phosphate buffer, pH 7.0 and 10 mM EDTA overnight at 37°C, followed by removal of color with 70% ethanol.

Measurement of germination rate, root elongation and fresh weight

To examine the germination rate under salt stress, extra NaCl was added to the normal B5 media at concentrations of 0, 100 or 150 mM. At least 100 seeds were used per treatment and germinated seeds were counted daily.

For root elongation assays, seedlings were germinated on B5-agar plates for 5 d. Then 30 seedlings with a similar growth status were transferred onto new agar media supplemented with 0, 100 or 150 mM NaCl or 200 mM mannitol, and grown vertically under normal conditions for another 9 d. Root elongation was monitored and measured at 3 d intervals. All 30 seedlings were collected and weighed together after the 9 d root growth assay in every treatment, and the average fresh weight per seedling was calculated.

For observation of growth, 5-day-old seedlings in a similar condition were transferred onto B5 media with 300 mM NaCl. Growth status was distinguished as three categories: normal, subhealthy and dead. The percentage of seedlings with each status was counted at 2 d intervals for 10 d.

Determination of Chl

Plants were grown in soil under normal conditions for 3 weeks and watered with liquid culture solutions. They were then watered with the same culture solutions with or without an extra 300 mM NaCl for another week. For each line, rosette leaves at the same position of ~20 plants were collected. A 150 μl aliquot of fresh weight of tissue was extracted with 20 ml of 80% acetone by incubation at 4°C in darkness for 12 h. Absorbance was measured using an UV-2550 UV-VIS spectrophotometer (SHIMADZU) at 663 and 645 nm. The concentration of total Chl was calculated from the sum of Chl a and b by the classic formula given previously (Arnon 1949).

Determination of proline and anthocyanin content

The same tissues collected for Chl determination were used for measuring the content of proline and anthocyanin. The free proline content in plant tissues was determined based on the photometric method (Troll and Lindsley 1955). A 200 μl aliquot of fresh weight of tissues was extracted with 3 ml of sulfosalicylic acid by boiling for 4 h, then cooled and shaken with permutit for...
The weight was determined and they were extracted in 10 ml of deionized water described previously (Mancinelli and Schwartz 1984).

The absorbance of the extracts, clarified by filtration, was measured at 530 and 657 nm. The formula

\[ A_{530} = 0.25 A_{657} \]  

was used to eliminate the contribution of Chl and its degradation products to the absorption at 530 nm, as described previously (Mancinelli and Schwartz 1984).

Determination of plant relative sodium content

Ten-day-old seedlings were transferred to new B5 agar medium with or without 200 mM mannitol or 200 mM NaCl for 6 h. Seedlings of each line were harvested, and were rinsed three times with 5 mM CaCl2 for 5 min, followed by deionized water three times for 5 min each. Seedlings were dried, their dry weight was determined and they were extracted in 10 ml of deionized water by boiling for 2 h. The extraction was filtered and the volume was set to 10 ml. The Na+ concentration was determined using an atomic absorption spectrophotometer (5 Series AA Spectrometer, Thermo Electron Corporation) with a standard curve made by standard Na+ solutions, and the relative sodium content was calculated using the data determined (mg g⁻¹ DW).

Image and statistical analysis

Photographic images were taken using a digital camera (Canon EOS 60D) and further processed by Adobe Photoshop. Quantitative image analysis for gene expression and root elongation was performed with ImageJ software (http://rsbioinfo.nh.gov/ij/) and processed by Microsoft Excel. Data were analyzed by one-way analysis of variance (ANOVA) with SPSS 17.0.0 (SPSS Inc.). All experiments were repeated three times and the means were compared by Duncan’s multiple range test. Different letters on the histograms indicate that the means were statistically different at the P < 0.05 level.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


