Differential expression of miRNAs in response to salt stress in maize roots

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• Background and Aims Corn (Zea mays) responds to salt stress via changes in gene expression, metabolism and physiology. This adaptation is achieved through the regulation of gene expression at the transcriptional and post-transcriptional levels. MicroRNAs (miRNAs) have been found to act as key regulating factors of post-transcriptional gene expression. However, little is known about the role of miRNAs in plants’ responses to abiotic stresses.

• Methods A custom µparaflo™ microfluidic array containing release version 10.1 plant miRNA probes (http://microrna.sanger.ac.uk/) was used to discover salt stress-responsive miRNAs using the differences in miRNA expression between the salt-tolerant maize inbred line ‘NC286’ and the salt-sensitive maize line ‘Huangzao4’.

• Key Results miRNA microarray hybridization revealed that a total of 98 miRNAs, from 27 plant miRNA families, had significantly altered expression after salt treatment. These miRNAs displayed different activities in the salt response, and miRNAs belonging to the same miRNA family showed the same behaviour. Interestingly, 18 miRNAs were found which were only expressed in the salt-tolerant maize line, and 25 miRNAs that showed a delayed regulation pattern in the salt-sensitive line. A gene model was proposed that showed how miRNAs could regulate the abiotic stress-associated process and the gene networks coping with the stress.

• Conclusions Salt-responsive miRNAs are involved in the regulation of metabolic, morphological and physiological adaptations of maize seedlings at the post-transcriptional level. The miRNA genotype-specific expression model might explain the distinct salt sensitivities between maize lines.

Key words: Salt stress, Zea mays, microRNA, microarray, transcription regulation, Zea mays.

INTRODUCTION

Salt stress is one of the most serious abiotic stresses of crop plants worldwide. To cope with high salt stress in their sessile lifestyle, crop plants have evolved a considerable degree of developmental plasticity, including adaptation via cascades of molecular networks. One of the most obvious features of the adaptation to salt tolerance are changes in gene expression profiles for genes involved in a broad spectrum of biochemical, cellular and physiological processes such as energy metabolism, signal transduction, transcription, protein biosynthesis and decay, membrane trafficking and photosynthesis (Vinocur and Altman, 2005). In addition, post-transcriptional regulation also plays a role in the plant salt response. Borsani et al. (2005) found that natural cis-antisense gene pairs initiated nat-siRNA (natural antisense small interfering RNA) formation, and the nat-siRNA then guided the cleavage of the P5CDH [Δ(1)-pyrroline-5-carboxylate dehydrogenase] transcript, which is a salt stress-related functional gene.

MicroRNAs (miRNAs) are approx. 21-nucleotide (nt) non-coding RNAs that play critical roles in gene expression regulation at the post-transcriptional level. In plants, cleavage of the target mRNA appears to be the prevalent method of post-transcriptional regulation, yet Brodersen et al. (2008) provided evidence that plant miRNA-guided silencing has a widespread translational inhibitory component. Plant miRNA-guided gene regulation has been shown to be involved in multiple developmental processes including organ polarity (Bowman, 2004), leaf growth (Chuck et al., 2007a), sex determination (Chuck et al., 2007b) and male or female sterility (Millar and Gubler, 2005). The observation that some plant miRNAs respond to stress conditions and some miRNA targets are stress-related genes suggests that miRNAs may play important roles in the plant stress response (Phillips et al., 2007). Several stress-specific miRNAs have also been identified in model plants under various biotic and abiotic stress conditions, including nutrient deficiency (Fujii et al., 2005), drought (Zhao et al., 2007), cold (Zhou et al., 2008), high salinity (Sunkar et al., 2008), bacterial infection (Navarro et al., 2006), UV-B radiation (Zhou et al., 2007) and mechanical stress (Lu et al., 2005).

Microarray technology has been applied in the high-throughput detection of gene expression and it has proved a useful tool in miRNA expression assays (Liu et al., 2008). A microarray-based search was undertaken to discover submergence stress-responsive miRNAs in maize roots (Zhang et al., 2008). High salinity is a serious abiotic stress for maize seedlings, and miRNA-guided post-transcriptional regulation might be involved in the response by the salt-shocked maize seedlings. In this study, a custom µparaflo™ microfluidic array (LC Sciences, Houston, TX) containing release version 10.1 plant miRNA probes (http://microrna.sanger.ac.uk/; Griffiths-Jones et al., 2008) was used to discover differentially expressed miRNAs between two maize inbred lines with
distinct salt sensitivities. The results indicated that only a minority of the differentially expressed miRNAs were uniformly regulated between the two maize lines; the vast majority of the differentially expressed miRNAs show a genotype-specific expression model. These miRNAs potentially play vital roles in the morphological and metabolic adaptation response in salt-shocked root cells of maize, and the genotype-specific expression model may explain the distinct salt sensitivities between the two maize lines.

MATERIALS AND METHODS

Plant treatment and RNA isolation

Seeds of ‘NC286’ (a salt-tolerant maize, Zea mays, inbred line) and ‘Huangzao4’ (a salt-sensitive maize line) were used in this study. For each inbred line, 60 uniform seeds were surface-sterilized in 70% (v/v) ethanol for 15 min, and then rinsed several times with sterile distilled water. The seeds were sown in sterile sand irrigated with sterile distilled water. The seeds were allowed to germinate in the incubator with a 16 h photoperiod at an optimal temperature regime of 24/21°C (light/dark). Uniform seedlings with two leaves were transferred into 1 x Hoagland's nutrient solution (supplied with 4 mL L⁻¹ Fe-sequestrene, 6 mM K⁺ and 4 mM Ca²⁺) and grown hydroponically with aeration. When the seedlings developed three leaves, they were transferred into either a nutrient solution containing 200 mM NaCl for salt stress treatment or the nutrient solution without NaCl, as a control. Plants were treated at 08 00 h, and roots of ten plants were harvested at 0-5, 5 and 24 h after salt stress; the roots of untreated control were also harvested at the corresponding time points. The collected roots were immediately rinsed several times with sterile distilled water. The seeds were surface-sterilized in 70 % (v/v) ethanol for 15 min, and then rinsed several times with sterile distilled water. The seeds were allowed to germinate in the incubator with a 16 h photoperiod at an optimal temperature regime of 24/21°C (light/dark). Uniform seedlings with two leaves were transferred into 1 x Hoagland’s nutrient solution (supplied with 4 mL L⁻¹ Fe-sequestrene, 6 mM K⁺ and 4 mM Ca²⁺) and grown hydroponically with aeration. When the seedlings developed three leaves, they were transferred into either a nutrient solution containing 200 mM NaCl for salt stress treatment or the nutrient solution without NaCl, as a control. Plants were treated at 08 00 h, and roots of ten plants were harvested at 0-5, 5 and 24 h after salt stress; the roots of untreated control were also harvested at the corresponding time points. The collected roots were immediately frozen in liquid nitrogen and ground into a fine powder. The total RNA from each sample was extracted with Trizol reagent (Invitrogen, Carlsbad, CA).

miRNA microarray assay

miRNA microarray assays were performed by LC Sciences. The custom µparaflo™ microfluidic chip contained 653 unique plant miRNAs of release version 10.1, representing 877 miRNAs from 17 plant species (http://microrna.sanger.ac.uk/; Griffiths-Jones et al., 2008). Each chip contained five repetitions of each probe. The 877 miRNAs comprised 154 from Arabidopsis thaliana, 115 from Oryza sativa, 187 from Physcomitrella patens, 100 from Populus trichocarpa, 43 from Zea mays and 278 from 12 other plant species. For microarray quality control, four controls were used. Maize 5S rRNA served as the internal reference control; and PUC2-20B, an artificial non-homologous nucleic acid, was used as an external positive control. The perfect match and single-base mismatch motifs via PlantCARE. To validate the precision of the TSS (transcription start site) may be as little as 50 nt and as much as 2.5 kb upstream of the pre-miRNA sequences in the transcript (Bracht et al., 2004; Megraw et al., 2006). To discover maize salt stress-responsive miRNA genes, each pre-miRNA sequence downloaded from http://microrna.sanger.ac.uk/ was aligned to the maize genome sequence (http://www.maizesequence.org). About 2500 bp of upstream sequences of the pre-miRNAs were checked by PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/; Lescot et al., 2002), the −75 CAAT-box and −30 TATA-box were recognized as the mile-stones to confirm the TSS. The 1500 bp DNA sequence upstream of the TSS was truncated to identify cis-acting motifs via PlantCARE. To validate the precision of the TSS prediction and promoter analysis, 5’ RACE (rapid amplification of cDNA ends) was undertaken to obtain the TSS, and primers of the upstream sequences were then designed to amplify the promoter regions of the miRNA genes. About 1000 bp of DNA sequence upstream of the TSS of each miRNA gene was amplified and sequenced to validate the prediction of the promoter sequences. The Supplementary Information (Table S1C, available online) shows the primers for five miRNA gene promoters.
In plants, miRNAs recognize their target mRNAs by perfect or near-perfect base pairing. Computational sequence similarity algorithms were developed to identify potential miRNA targets. For the prediction of plant miRNA target miRNAs, a web-based computing system, miRU (Zhang et al., 2005), was queried by mature miRNA sequences. All potential sequences complementary to the query with limited mismatches were selected. The number of mismatches was limited to no more than 3 nt, indels were no more than 1 nt, and fewer than five G–U pairs were allowed. The predicted target expressed sequence tags (ESTs) were aligned and searched by BLAST for functional annotation.

Validation of the mature miRNA expression profile via stem–loop RT–PCR

The expression profiles of seven salt-responsive mature miRNAs were assayed by stem–loop reverse transcription-PCR (RT–PCR). A 200 ng aliquot of total RNA was used for the initiation of the reverse transcription reaction. The stem–loop reverse transcription primers were designed following the method described by Chen et al. (2005) and Varkonyi-Gasic et al. (2007). Briefly, 6 nt tips pairing with the mature miRNA 3’ end were linked to a self-looped sequence (GTCGTATCCAGTGCAGGGTCCGAGGTATTCCGACTGGATAAGC) to make up the stem–loop RT-PCR primers; the primers bind to the 3’ portion of miRNA molecules, initiating reverse transcription of the mature miRNA. The reverse transcription product is amplified using a miRNA-specific forward primer and a universal reverse primer. The stem–loop reverse transcription reactions were performed by MMLV reverse transcriptase (Toyobo, Osaka, Japan) according to the supplier’s manual. PCR primers were then added to perform the PCR. One of the uniformly expressed miRNAs, miR172, was used as the inner control for stem–loop RT-PCR. Supplementary Table S1A shows the sequences of stem–loop RT primers and miRNA-specific PCR primers.

Validation of the miRNA target gene expression profiles by RT-PCR

The expression profiles of the predicted target genes were assayed by RT-PCR. A 10 µg aliquot of total RNA was used for initiating the reverse transcription reaction. The oligo(dT) primer was added and incubated with the total RNA. The reverse transcription reaction was performed by MMLV reverse transcriptase (Toyobo, Osaka, Japan) according to the supplier’s manual. The target gene primers were then added to perform the PCR. A maize housekeeping gene, γ-tubulin, was used as the inner control for RT-PCR. Supplementary Table S1B shows the primer sequences of the target genes and the γ-tubulin gene.

RESULTS

Salt-stress-responsive miRNAs in maize roots

The microarray data showed that the expression profiles of many miRNAs changed in response to salt stress in maize roots. These miRNAs were identified from diverse families of plant miRNAs. Mature miRNAs are conserved among plant species; members of the same miRNA families differ from each other by only 1–3 nt, or they are identical. The differences exist in the pre-miRNA sequences. Thus, cross-hybridization might have occurred in the microarray between members of the same miRNA family, or between homologous sequences across species. To refine the differentially expressed miRNAs, miRNA families where many members showed changed expression profiles, especially whose members showed similar expression profiles, were considered as salt stress-responsive miRNA families. miRNAs with similar expression profiles reflected the expression of the family counterparts in maize. A total of 98 miRNAs, about 15 % (98/653) of the probes on the microarray, were identified as putative salt-responsive miRNAs at the 1 % significance level (Table 1). A total of 77 out of the 98 miRNAs aligned with 55 members of ten maize miRNA families, while the other miRNAs corresponded to several members of miRNA families from another six plant species, including osa-miR528/529, ath-miR395/398/827/854, ptc-miR474, ppi-miR395/477/529/535/894/896/903/1026, tae-miR1125 and smo-miR1082. The results shown in Fig. 1 indicate that, during the salt stress process, the differentially expressed miRNAs were transcriptionally regulated at different time points in the two maize lines. By stem–loop RT-PCR, the expression profiles of seven salt-responsive miRNAs were generated in the two maize inbred lines (Fig. 2B). The results are consistent with the microarray data (Fig. 2A).

Different expression profiles of salt-stress-responsive miRNAs between inbred lines

At three time points during the salt shock, miRNAs showed different expression profiles within the two inbred lines (Fig. 1). There are both congruously and differently regulated miRNAs, and inbred line-specific miRNAs were also detected. The differentially expressed miRNAs were classified into four groups using their expression profiles between the two maize inbred lines as a basis. Only six miRNAs (from three miRNA families, ptc-miR474, ppi-miR395 and osa-miR528) were detected to be uniformly regulated at all three time points in the two maize inbred lines. The vast majority of the differentially expressed miRNAs showed different expression patterns either among the three time points, or between the two maize lines. miRNAs from eight families showed the same expression trends, yet were regulated at different time points, most of which exhibited a delayed expression pattern in the salt-sensitive maize line ‘Huangzao4’. Nine miRNA families have different expression trends during the salt stress period between the two lines. There exists another group of salt stress-responsive miRNAs from ten families, which could only be detected in the salt-tolerant maize line ‘NC286’. The results strongly suggested that the different miRNA regulation patterns might contribute to and partly explain the distinct salt sensitivities between the two maize lines.

Potential target genes of salt-stress-responsive miRNAs

miRNAs regulate gene expression by a perfect or near-perfect binding with their target miRNAs and in turn guide
### Table 1. Salt-stress-responsive miRNA families and members

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Key cis-acting elements within the promoter regions of miRNA genes

All of the promoter regions of 60 maize counterpart salt stress-responsive miRNA genes were queried by PlantCARE. Five of the miRNA genes promoters were further validated by 5′ RACE and PCR amplification. The Supplementary Information (Figure S1, available online) shows the sequences of the five promoters. The experimental results showed a perfect consistency with the prediction. Stress-responsive cis-motifs were found in the 5′-upstream area (1.5 kb predicted promoter regions) of these miRNA genes (Supplementary Information, Table S2). The cis-elements include ABRE (ABA-responsive element), MBS (MYB binding site), TCA (salicylic acid-responsive element), HSE (heat shock-responsive element), LTR (low temperature-responsive element), GC-motif, ARE (anaerobic-response element) and GARE (gibberellin-responsive element). Each of the salt stress-responsive miRNAs has more than one stress-responsive cis-acting element in their promoter regions, indicating that development processes, including energy metabolism, signal transduction, transcriptional regulation and cell defence. The expression profiles of seven of the target genes were validated by RT-PCR, and show a negative correlation with their corresponding zma-miRNAs (Fig. 2C). Using the functional annotations of the predicted target genes, they could be classified into four categories. Similarly to previously reported data in model plants, many miRNAs detected in the maize salt stress response directly targeted transcription factors (TFs) involved in plant development and organ formation. The TFs Myb, NAC1 and homeodomain-leucine zipper protein (HD-ZIP) were predicted as the targets of zma-miR159a/b, zma-miR164a/b/c/d and zma-miR166l/m, respectively. The results were consistent with those reported in arabidopsis and rice (Jones-Rhoades and Bartel, 2004). Other TFs predicted as the miRNA targets, such as MADS-box proteins and zinc-finger proteins, have been reported as salt stress-responsive factors in plants (Fang et al., 2006; Xu et al., 2008). Several targets of the miRNAs, including auxin response factor 8, ethylene-responsive element-binding factor and GAMYb, are involved in phytohormone signal cascades. In addition, several targets of miRNAs encode proteins that play roles in diverse metabolic pathways or are involved in various physiological processes. Many of the predicted targets, such as NAPD-dependent malic enzyme (NADP-ME) and cytochrome oxidase, were previously reported as salt stress-responsive functional genes in plant cells (Yan et al., 2005; Cheng and Long, 2007). Interestingly, an ARGONAUTE1 (AGO1) miRNA and a DCL1 gene, which encode key factors involved in miRNA processing, were predicted as the targets of zma-miR168a/b and zma-miR162, respectively. This result indicated that feedback regulation might play a role in miRNA activity under salt-stressed conditions in maize.

FIG. 1. Clustering of differently expressed miRNA members under salt stress at three time points in two maize inbred lines. (A) Significantly detected miRNAs in the salt-sensitive maize line ‘Huangzao4’; (B) significantly detected miRNAs in the salt-tolerant maize line ‘NC286’. The three time points are shown at the top, and salt-responsive miRNAs are listed on the right. Green indicates salt-repressed, whereas red indicates salt-induced miRNAs.
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miR162

miR164

miR167

miR168

miR395

miR396

microRNA profiles in ‘Huangzao4’

microRNA targets in ‘Huangzao4’

SBP domain-protein

RNA helicase CAF protein

NAC

ARF8

AGO1

ATP sulfurylase

Cytochrome oxidase

γ-tubulin

microRNA targets in ‘NC286’

SBP domain-protein

RNA helicase CAF protein

NAC

ARF8

AGO1

ATP sulfurylase

Cytochrome oxidase

γ-tubulin

Ding et al. — Maize salt-stress-responsive microRNAs
Table 2. Predicted target genes and their protein annotation

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These miRNAs are involved in regulation of cascades of various biotic and abiotic responses. As shown in Supplementary Table S2, the three most frequently appearing cis-acting motifs of the salt stress-responsive miRNA genes are MBS, ARE and ABRE. In addition, the miRNA members in the same miRNA family often share the same cis-acting motifs in their promoter regions, suggesting that the miRNA members of the same miRNA family may respond to the same biotic, abiotic or phytohormone stimuli.

**DISCUSSION**

Specifically and non-specifically regulated miRNAs responding to salt stress

Some miRNAs have been shown to be stress regulated and could be involved in cell responses to abiotic stresses such as salinity, cold and dehydration (Zhao et al., 2007; Sunkar et al., 2008; Zhou et al., 2008). Some miRNA target genes are stress-responsive TFs or functional genes (Yan et al., 2005; Fang et al., 2006; Cheng and Long, 2007; Prashanth et al., 2008; Xu, 2008), indicating that miRNA-dependent post-transcriptional regulation may play a role in the plant stress response. Stress response-specific miRNAs were observed in various studies. The miRNA ath-miR319c was specifically upregulated by cold but not by ABA, dehydration or salt shock (Sunkar and Zhu, 2004). The miRNA osa-miR169 g was confirmed as the only miRNA induced by drought in rice (Zhao et al., 2007). Genotypic specificity is also evident, since different genotypes express the same miRNA in the same tissues, but at different levels (Mica et al., 2006). Sunkar et al. (2007) reviewed the plant small RNAs responsive to abiotic stresses and nutrient deprivation and speculated that small RNAs that are positively regulated by stress might target negative regulators of stress tolerance for enhanced suppression; in contrast, small RNAs that are suppressed during stress are likely to target positive regulators of stress tolerance, resulting in the accumulation of gene products. In the present research, salt-responsive miRNAs were detected in two maize inbred lines that have distinct salt sensitivities. There are uniformly regulated miRNAs, yet a majority of the responding miRNAs were differentially regulated between the two maize lines (92/98). The differentially regulated miRNAs were either detected only in one inbred line or had distinct expression trends. The similarly regulated miRNAs may represent the fundamental mechanism of adapting to salt shock, and the differentially regulated miRNAs might explain the distinct salt sensitivities between the two maize inbred lines. Members of the miR474 and miR395 families were found to be similarly regulated between the two maize lines; they were both upregulated, indicating that they might target negative regulators of salt tolerance, resulting in enhanced suppression of these factors. Members of the miR396 family were found to be downregulated in both lines, yet at different time points. This result indicated that accumulation of different levels of positive regulators during the stress process might result in different salt sensitivities among maize inbred lines. Under salt stress conditions, global gene expression profiles were changed, resulting in metabolic and physiological alterations. The differential expression of the miRNAs within the two maize inbred lines showed that there were both non-specific and specific responses of miRNAs under salt shock in maize root cells.

Non-specific cascades controlled by miRNAs

miRNAs are known to silence genes post-transcriptionally by guiding target mRNAs for degradation or by repressing translation (Sunkar et al. 2007). NADP-ME is a key enzyme that catalyses the oxidative decarboxylation of L-malate to yield pyruvate, CO₂ and NADPH in the presence of a divalent cation (Cheng and Long, 2007). In plants, NADP-ME...
AGO1 indicated negative feedback regulation in the miRNA for AGO1 homeostasis. The co-adjustment of miR168 and co-regulation of miR168 and AGO1 genes and post- the arabidopsis ARGONAUTE family involved in the AGO1-catalysed mRNA cleavage. It is the only member of 'Huangzao4' at the 0.5 h time point. Correspondingly, inbred line 'NC286' and repressed in the salt-sensitive line zma-miR168 family were induced in the salt-tolerant maize salt stress-responsive miRNA; the members of the (Juarez et al., 2006). miR168 was found to cleave rld1 mRNA and alter leaf polarity (D. W. Kim et al., 2007). Thus, the scavenging of ROS may be another salt stress strategy conserved among maize inbred lines.

miRNA affect salt sensitivities of maize lines

AGO1 encodes the RNA slicer enzyme of the miRNA pathway and is regulated by miR168-programmed, AGO1-catalysed mRNA cleavage. It is the only member of the arabidopsis ARGONAUTE family involved in the miRNA-directed mRNA cleavage processes. Transcriptional co-regulation of miR168 and AGO1 genes and post-transcriptional stabilization of miR168 by AGO1 are required for AGO1 homeostasis. The co-adjustment of miR168 and AGO1 indicated negative feedback regulation in the miRNA pathway (Vaucheret et al., 2006), miR168 was found to be a salt stress-responsive miRNA; the members of the zma-miR168 family were induced in the salt-tolerant maize inbred line ‘NC286’ and repressed in the salt-sensitive line ‘Huangzao4’ at the 0.5 h time point. Correspondingly, AGO1 mRNA was suppressed in ‘NC286’ and accumulated in ‘Huangzao4’. Since AGO1 is a key miRNA pathway regulator, the difference in AGO1 mRNA expression at the early phase under salt shock may induce further changes of numerous miRNA activities. In general, during the early period under salt shock, miRNA function was enhanced in ‘Huangzao4’ and reduced in ‘NC286’. The watershed for the salt stress response in plants is 24 h; after this time point the expression profiles of various miRNAs may change. miR168 was detected to be downregulated in both lines at 24 h, resulting in AGO1 mRNA accumulation and enhanced miRNA functions. This might explain why there are so many changes in expression of miRNAs after 24 h of salt stress.

It has been reported that many miRNA target genes were miRNAs of TFs, indicating an upstream regulation of miRNAs during the development processes and environmental response. Some miRNAs, including miR166, miR159, miR156 and miR319, showed altered expression profiles in at least one salt-shocked maize line. They were shown to regulate the levels of TF transcripts, which in turn affected the levels of TF proteins. The maize Rolled leaf 1 (rld1) gene is a homologue of the arabidopsis HD-ZIP gene. In maize, miR166 was found to cleave rld1 mRNA and alter leaf polarity (Juarez et al., 2004). The ath-miR166-mediated cleavage of HD-ZIP was found to regulate the pattern of vasculature and the establishment and maintenance of abaxial–adaxial polarity in lateral organs (Williams et al., 2005). Other than their involvement in leaf polarity regulation, the HD-ZIP family members were also reported to be induced by various stress conditions, including drought and phytohormones (Agalou et al., 2008; Dai et al., 2008). Shin et al. (2004) reported that Athb-12, a HD-ZIP from arabidopsis, could suppress the Na⁺-sensitive phenotype. Expression of Athb-12 dramatically enhanced NaCl tolerance. Athb-12 can regulate the expression of NaCl-inducible PMR2A, which encodes a plasma membrane Na⁺-ATPase involved in Na⁺ transport. Downregulation of miR166 in the salt-tolerant maize line ‘NC286’ after 5 h of salt shock indicated that an HD-ZIP, perhaps rld1, was involved in maize salt tolerance. The downregulation of miR166 in the salt-tolerant maize line and the upregulation of the target HD-ZIP transcripts might enhance the salt tolerance. The TCP domain is a plant-specific DNA-binding domain found in proteins from a diverse array of species. TCP TFs direct the developmental process of plant leaf size and form, and flower symmetry (Busov et al., 2008). The mRNA of the TCP TFs were targets of miR319, which was downregulated in the salt-tolerant maize line ‘NC286’ after 5 h and in the salt-sensitive maize line ‘Huangzao4’ after 24 h of salt shock. The downregulation of miR166 and miR319 after a period of salt shock in the maize inbred lines indicated an induction of their target TF transcripts and resulted in enhanced salt tolerance. The unique regulation of the miRNAs and their target TF transcripts might explain the different salt sensitivities between the two maize lines.

The regulation of miR166 and miR319 after 5 and 24 h of salt shock suggested that, although the miR-TF model is involved in the salt stress response, the salt response actions were not initiated by regulation of the TFs. At the 0.5 h time point, miR167 and miR164 were found to be downregulated in ‘NC286’, hinting that these miRNAs might be involved in the initiation of receipt of the salt stress signal. ARF8, an auxin-responsive factor gene, was predicted as the target of zma-miR167. Auxin response is regulated by various positive and negative feedback mechanisms as well as environmental signals. ARFs regulate the transcription of early auxin-responsive genes, including the AUX/IAA genes (Ulmasov et al., 1997), whereas AUX/IAA proteins interact with ARFs and repress their activities (Tiwari et al., 2003). Auxin induces targeted ubiquitinylation/degradation of specific AUX/IAA proteins (Gray et al., 2001) and frees ARFs from repression by AUX/IAA proteins. The accumulation of ARF transcripts resulting from the downregulation of miR167 might enhance the auxin response and thus enhance shoot and leaf development. NAC-domain proteins, named from a conserved domain originally associated with the NO APICAL MERISTEM (NAM) phenotype, were reported to be salt induced in many species (Hu et al., 2006; S. G. Kim et al., 2007). NAC-domain proteins are unique to plants, and their transcripts are the predicted targets of zma-miR164. Xie et al. (2000) reported that in arabidopsis, 35S::NAC1-overexpressing lines were bigger, with larger leaves, thicker stems and more abundant roots than the wild type. They thought that NAC1 might be an early auxin-responsive gene,
and confirmed that NAC1 was located downstream of TIR1 and upstream of AIR3 and DBP in transmitting the auxin signal to the AIR3 gene to promote lateral root development. TIR1 is likely to regulate NAC1 at the transcriptional level, perhaps through auxin-dependent degradation of a negative regulator of NAC1. The placement of lateral roots is not predetermined but it is strongly influenced by endogenous auxin levels and exogenous environmental conditions. It was observed in this study that the lateral roots flourished more in ‘NC286’ than in ‘Huangzao4’; this might have resulted from the early accumulation of auxin-responsive factors. At the early stage of salt stress, the salt-tolerant maize lines might change their morphological characteristics to enhance root and shoot development, thus accumulating more biomass to counteract the wastage brought on by salt shock.

Here a model has been proposed for the molecular mechanisms of the response of maize root cells to salt shock using the changed expression profiles of miRNA and subsequent target transcripts as a basis. The responsive miRNAs show a transitional expression model. Figure 3 shows the proposed regulation cascades after 0.5 h salt shock in ‘NC286’ root cells. This study indicates that the salt-responsive miRNAs are involved at post-transcriptional levels in the regulation of the metabolic, physiological and morphological processes of maize seedlings under salt-shocked conditions.

SUPPLEMENTARY INFORMATION

Supplementary information is available online at www.aob.oxfordjournals.org/ and consists of the following. Table S1(A), the stem–loop reverse transcription and PCR primer sequences; Table S1(B), the accessions of target genes and primers for RT-PCR; Table S1(C), PCR primers for miRNA promoter amplification. Table S2, stress-responsive key cis-elements found in zma-miRNA promoters. Figure S1, five of the maize miRNA promoter sequences obtained by PCR amplification and sequencing.

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