Transgenic creeping bentgrass overexpressing Osa-miR393a exhibits altered plant development and improved multiple stress tolerance

Junming Zhao1,2,†, Shuangrong Yuan1,†, Man Zhou3,†, Ning Yuan1, Zhigang Li1, Qian Hu1, Frank G. Bethea Jr.4, Haibo Liu4, Shigui Li5, and Hong Luo1,6,*

1Department of Genetics and Biochemistry, Clemson University, Clemson, SC, USA
2Animal Science and Technology College, Sichuan Agricultural University, Chengdu, Sichuan, China
3College of Natural, Applied and Health Sciences, Wenzhou Kean University, Wenzhou, Zhejiang, China
4Department of Plant and Environmental Sciences, Clemson University, Clemson, SC, USA
5Rice Research Institute, Sichuan Agricultural University, Chengdu, Sichuan, China

Received 16 November 2017; revised 19 May 2018; accepted 2 June 2018.
*Correspondence (Tel: 1 864 656 1746; fax 1 864 656 0393; emails hluo@clemson.edu (H.L.); Ishigui_li@263.net (S.L.))
†These authors contributed equally to this work.

Keywords: miR393, plant development, salt tolerance, drought tolerance, heat tolerance, turfgrass, transgenics, microRNA.

Summary

MicroRNA393 (miR393) has been implicated in plant growth, development and multiple stress responses in annual species such as Arabidopsis and rice. However, the role of miR393 in perennial grasses remains unexplored. Creeping bentgrass (Agrostis stolonifera L.) is an environmentally and economically important C3 cool-season perennial turfgrass. Understanding how miR393 functions in this representative turf species would allow the development of novel strategies in genetically engineering grass species for improved abiotic stress tolerance. We have generated and characterized transgenic creeping bentgrass plants overexpressing rice pri-miR393a (Osa-miR393a). We found that Osa-miR393a transgenics had fewer, but longer tillers, enhanced drought stress tolerance associated with reduced stomata density and denser cuticles, improved salt stress tolerance associated with increased uptake of potassium and enhanced heat stress tolerance associated with induced expression of small heat-shock protein in comparison with wild-type controls. We also identified two targets of miR393, AsAFB2 and AsTIR1, whose expression is repressed in transgenics. Taken together, our results revealed the distinctive roles of miR393/target module in plant development and stress responses between creeping bentgrass and other annual species, suggesting that miR393 would be a promising candidate for generating superior crop cultivars with enhanced multiple stress tolerance, thus contributing to agricultural productivity.

Introduction

In recent years, due to the dramatic changes in climate and environment, plants are constantly challenged by a broad range of environmental stresses, which pose serious threats to crop productivity (Suzuki et al., 2014). Abiotic stresses such as drought, high salinity and temperature fluctuations lead to more than 50% yield loss in major crops worldwide each year (Bary et al., 2000; Lobell et al., 2011; Shao et al., 2009). Furthermore, abiotic stress can also adversely affect plant defence systems and increase plant susceptibility to pathogen infections (Atkinson and Urwin, 2012; Goel et al., 2008; Luck et al., 2011). Thus, plants are most likely to suffer from various environmental stresses occurring concurrently. It is estimated that almost two times of current crop production is required to meet the ever-increasing population by 2050, whereas the arable land is not expected to expand dramatically (FAO, 1996). Therefore, it is critical to breed multiple stress-tolerant crops.

To cope with environmental stresses, plants have evolved complex defence mechanisms involving multiple components, including pathways in signal perception and transduction, transcriptional regulation and downstream stress-responsive gene expression (Chaves et al., 2003; Xiong et al., 2002). A large number of stress-responsive genes that have been identified can be further classified into two classes. The first class of stress-responsive genes encodes functional proteins that directly contribute to the protection of plant cells from stresses, such as detoxification enzyme, water channel, osmolytes, chaperones and late embryogenesis abundant (LEA) proteins. The second class includes genes for regulatory proteins such as transcription factors (TFs), protein kinases, phosphatases and other signalling molecules as well as noncoding RNAs (Shinozaki and Yamaguchi-Shinozaki, 2007; Shinozaki et al., 2003; Zhou and Luo, 2013). Manipulating specific master regulatory genes in transgenic plants have been demonstrated to be effective in engineering a broad-spectrum stress tolerance (Chen et al., 2014).

MicroRNAs (miRNAs) are small regulatory RNAs that act at the post-transcriptional level to guide target mRNA cleavage or function for translation inhibition based on the complementary sequence between miRNAs and their targets (Bartel, 2004; Beauclair et al., 2010). An increasing number of studies on plant miRNAs have demonstrated that they are promising candidates for enhancing multiple stress tolerance in plants, for they can target multiple genes, most of which are TFs, already known to be good candidates for crop genetic engineering (Rhoades et al., 2002; Wang et al., 2016). Besides, miRNAs also regulate other stress mediators, such as protein kinases, phytohormone signalling components and reactive oxygen species (ROS).
scavenging enzymes (Bian et al., 2012; Raghuram et al., 2014; Sunkar et al., 2006). It is known that protein kinases play critical roles in multiple stress response (Sinha et al., 2011). Phytohormones (e.g. auxin, cytokines, gibberellins, abscisic acid, jasmonic acid, salicylic acid, ethylene and brassinosteroids) and ROS are also vital in signal transduction when plants encounter various adverse environmental conditions (Zhou et al., 2016). All these indicate that miRNAs are master regulators in plant response to various environmental cues and may have great potential in crop genetic engineering for enhanced plant performance under adverse environmental conditions. Indeed, this has been demonstrated for an increasing number of miRNAs, such as miR319, miR528, miR396 and miR408 (Chen et al., 2015a; Ma et al., 2015; Yuan et al., 2015; Zhou et al., 2013). Recently, a conserved miRNA, miR393 has been identified with the potential of mediating plant response to a variety of biotic and abiotic stresses. Overexpression of miR393 caused increased antioxidant resistance in Arabidopsis, but reduced drought and salinity tolerance in rice (Gao et al., 2011; Navarro and Jones, 2006; Xia et al., 2012). In rice and Arabidopsis, the miR393 family contains two family members, miR393a and miR393b. Although the two members have the same targets and are both involved in auxin perception regulation and cadmium stress response in rice (Ding and Zhu, 2009; Si-Ammour et al., 2011), they exhibit distinctive expression patterns. In rice, miR393a expressed mainly in the crown and lateral root primordial and coleoptiles tip, while miR393b expressed in the shoot apical meristem (Bian et al., 2012); the expression level of the miR393a changed under salinity and alkaline stresses, whereas that of the miR393b did not (Gao et al., 2011). In addition to stress responses, miR393 also plays a crucial role in plant growth and development, such as leaf development, root architecture, coleoptile elongation, stomatal development and flowering (Guo et al., 2016a; Si-Ammour et al., 2011; Vidal et al., 2010; Xia et al., 2012). Studies in Arabidopsis and rice showed that overexpression of miR393a increased shoot and tiller numbers, because auxin signalling was repressed through down-regulation of miR393 targets, F-box auxin receptors (AtTIR1, AtAFB1, AtAFB2 and AtAFB3 in Arabidopsis; OsTIR1 and OsAFB2 in rice) (Si-Ammour et al., 2011; Xia et al., 2012). Although the role of miR393 has been investigated in a number of plant species including switchgrass, a C4 warm-season perennial grass species (Liu et al., 2017), it has not been characterized in C3 cool-season perennial grasses. To further our knowledge about the functions of miR393, unravelling its role in plant response to multiple environmental stresses and the underlying molecular mechanisms, we used creeping bentgrass (Agrostis stolonifera L.), an environmentally and economically important C3 perennial turfgrass, to carry out our study.

In this study, we have generated and characterized transgenic creeping bentgrass plants overexpressing rice pri-miR393a (Osa-miR393a). We found that the transgenics have fewer, but longer tillers with enhanced drought, salt, and heat stress tolerance in comparison with wild-type controls. We also identified two targets of miR393a, AsAFB2 and AsTIR1, whose expression is repressed in the transgenics. Our results reveal the distinctive roles of miR393a/target module in plant development and stress responses between creeping bentgrass and other annual species and demonstrate the importance of miR393a as a promising candidate for manipulation in generating superior crop cultivars with enhanced multiple stress tolerance, thus contributing to agricultural productivity.

### Results

miR393 responds to salt, drought, and heat stresses and auxin treatment in creeping bentgrass

Previous studies demonstrated that miR393 is involved in plant responses to drought, salt stresses and auxin treatment in annual species (Chen et al., 2011, 2015b; Gao et al., 2011; Xia et al., 2012). In this study, we investigated its role in perennial grass species, creeping bentgrass. To this end, we first examined the spatial expression patterns of miR393 in creeping bentgrass. Stem-loop RT–qPCR analysis demonstrated that the mature miR393 is most abundant in leaves followed by stems, while the expression level of mature miR393 is relatively low in roots and flowers (Figure S1a). Next, we quantitatively analysed miR393 expression in response to salt, drought, and heat stresses and IAA treatment in leaves. Mature miR393 was found to be significantly induced by these abiotic stressors and auxin treatment (Figure S1b–e), in agreement with observations in Arabidopsis and rice (Chen et al., 2011, 2015b; Gao et al., 2011; Xia et al., 2012), suggesting that miR393 is also regulated by various abiotic stresses and hormone auxin in creeping bentgrass.

#### Generation of transgenic creeping bentgrass

**overexpressing the rice miR393 gene, Osa-miR393a**

miR393 response to various environmental cues and auxin treatment in turfgrass prompted us to further investigate its possible involvement in determining plant adaption to abiotic stresses. To this end, a miR393 overexpression construct was prepared and introduced into creeping bentgrass Penn A-4’ via Agrobacterium tumefaciens-mediated plant transformation. As shown in Figure S2a, the stem-loop precursor of rice Osa-miR393a gene was under the control of CaMV35S promoter and linked to a CaMV35S promoter-driving hygromycin resistance gene, Hyg. Multiple transgenic lines were identified through Hyg gene and Osa-miR393a amplification, respectively (Figure S2b,c). All lines showed transcription of the rice pri-miR393a (see examples in Figure S2c). Interestingly, two independent transgenic lines, TG3 and TG5, displayed significantly higher pri-miR393a transcription than others (Figure S2c) and exhibited extremely dwarf phenotype with early senescence and died unexpectedly (Figure S3a). It seems that the miR393-mediated plant development may be dose-dependent. High-dose miR393 would significantly impact plant development, resulting in severely dwarf phenotype, and even lethal in the worst-case scenario. Elevated transcripts of the mature Osa-miR393a in transgenic lines compared to wild-type control plants revealed by stem-loop RT–qPCR analysis further confirm the proper processing of the primary miRNAs sequence of the rice Osa-miR393a into mature miRNAs in creeping bentgrass (see examples in Figure S2d for normal type transgenics). It should be noted that contrary to that in TG2 and TG4, the accumulations of the pri-miR393 and the mature miR393 are not positively correlated in TG1 (Figure S3c,d). This phenomenon has also previously been observed for other miRNA genes in transgenics of different plant species, for example the primary and mature miR282 in Arabidopsis and the primary and mature miR393 in switchgrass (Liu et al., 2017; Yang et al., 2013). Presumably, the primary transcripts of the overexpressed miRNA (pri-miRNA) might be more efficiently processed into its mature form in some transgenic events, and therefore, the remaining pri-miRNA becomes much less than the mature miRNA. This higher level of mature miR393 accumulation

© 2018 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 17, 233-251
mir393a plants displayed significantly fewer, but longer tillers than wild-type controls (Figure 1a–c,i–k) both at the earlier (5 weeks old) and at later (10 weeks old) developmental stages (Figure 1l). We also found that transgenic plants have significantly more and longer internodes from each tiller (Figure 1d,k), wider leaves and larger stems than wild-type controls (Figure 1f, m,n). Histological analysis of transgenic and wild-type leaves and stems at the cellular level under microscope indicated that the numbers of vascular bundles in both leaves and stems were significantly increased in transgenics in comparison with wild-type controls (Figure 1g,h). This might attribute to the wide leaf and large stem phenotypes observed in transgenic plants.

To study the potential impact of miR393 on plant growth, we measured shoot and root biomass of 10-week-old wild-type and

Figure 1 Development of wild-type (WT) and transgenic (TG) plants. (a) Ten-week-old wild-type and transgenic plants initiated from a single tiller. Bar = 5 cm. (b) Tiller number of 10-week-old wild-type and transgenic plants initiated from a single tiller. (c) Close-up of the longest tillers from wild-type and transgenic plants. Bar = 5 cm. (d) All internodes from the representative longest tiller were sliced from top to bottom and displayed in order from left to right. Bar = 5 cm. (e) Root system of 10-week-old wild-type and transgenic plants initiated from a single tiller. Bar = 5 cm. (f) Top fully developed leaf from the representative tillers of wild-type and transgenic plants. Bar = 5 cm. (g) Cross-sectional images of wild-type and transgenic leaves. Bar = 200 μm. (h) Cross-sectional images of wild-type and transgenic stems. Bar = 200 μm. (i) Tiller number in wild-type and transgenic plants 5 and 10 weeks after initiation from a single tiller. (j) Shoot number in wild-type and transgenic plants 30, 60 and 90 days after initiation from a single tiller. (k) Statistical analysis of the longest tiller length between representative wild-type and transgenic plants. (l) Statistical analysis of biomass between representative wild-type and transgenic plants. (m) Statistical analysis of the leaf blade width between representative wild-type and transgenic plants. (n) Statistical analysis of the stem diameter between representative wild-type and transgenic plants. (o) Fully developed WT and TG lines (TG1, TG4) with ten tillers grown in cone-tainers were mowed weekly to the same height. Clipping fresh weight was measured at the end of 1st, 2nd and 3rd weeks. Data are presented as means (n = at least 5), and error bars represent SD. Asterisks indicate a significant difference between the wild-type and each transgenic line at *P < 0.05; **P < 0.01; and ***P < 0.001 by Student’s t-test.

© 2018 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 17, 233-251
transgenic plants initiated from a single tiller and the weekly clipping fresh weight. Although compared to wild-type controls, a decreased shoot biomass was observed in transgenic plants, the difference was nonsignificant (Figure 1i), suggesting that the increased tiller length in Osa-miR393a plants might compensate for the loss in biomass caused by decreased tillering. The shoot weekly clipping fresh weight in transgenic plants was significantly lower than that in wild-type controls (Figure 1o), indicating that the leaf biomass accumulation of the transgenic plants was slower than that of the control plants. However, the root biomass of Osa-miR393a plants was significantly lower than that of the wild-type controls (Figure 1i).

Overexpression of miR393 leads to improved salt tolerance in transgenic plants

Overexpression of miR393 decreases plant salt stress resistance in transgenic rice (Xia et al., 2012). This observation, together with our expression analysis of miR393 in creeping bentgrass responding to salinity stress (Figure S1b), prompted us to investigate what role miR393 plays in regulating plant salt stress response in perennial grasses. As shown in Figures 2a–c and S4a–c, when subjected to 250 mM NaCl treatment, significant tissue damage and hampered plant growth were observed 10 days after salinity stress in wild-type controls compared with transgenic plants, suggesting an improved salt tolerance in Osa-miR393a transgenics. To further elucidate the physiological mechanism of enhanced salt tolerance in Osa-miR393a plants, we investigated cell membrane integrity, water status and total chlorophyll content of transgenic lines in comparison with wild-type controls. While the rate of electrolyte leakage (EL) was similar in wild-type controls and two transgenic lines tested under normal growth conditions, it was significantly higher in wild-type plants than in transgenics after a 10-d salinity stress (Figure 2e). The result indicates a better cell membrane integrity maintained in Osa-miR393a transgenic lines than in wild-type controls exposed to high salinity. Salt stress also impacts water status in plants. The relative water content (RWC), a widely used parameter to monitor plant water status was found to be significantly higher in transgenics than in wild-type controls when subjected to salinity stress (Figure 2d), further supporting a correlation between enhanced high-salinity resistance and elevated levels of Osa-miR393a in transgenic lines. Malondialdehyde (MDA), an indicator of oxidative damage, is accumulated in plants with the development of salt stress (Halliwell and Gutteridge, 1989). We found that MDA content in wild-type and transgenic creeping bentgrass was similar under normal growth conditions (Figure 2f). Although it was induced dramatically upon NaCl application in both Osa-miR393a transgenics and wild-type controls, the accumulation of MDA in two transgenic lines was significantly less pronounced than that in wild-type controls (Figure 2f) and therefore less leaf damage in transgenic lines than in wild-type controls. Moreover, during salt treatment, two transgenic lines maintained significantly higher total chlorophyll contents than wild-type controls (Figure 2g), suggesting an improved photosynthesis in transgenics in comparison with wild-type controls, and thereby contributing to enhanced salt stress resistance.

Figure 2 Responses of wild-type controls (WT) and transgenics (TG) to salinity treatment. (a) Wild-type controls and three transgenic lines initiated from the same number of tillers were fully developed in cone-tainers for 10 weeks under normal conditions in growth room before salt stress application. (b) Performance of wild-type and transgenic plants subjected to 250 mM NaCl treatment for 10 days. (c) Performance of wild-type and transgenic plants 7 days after recovery from a 10-day salt treatment. (d) Relative water contents, (e) electrolyte leakage values, (f) MDA contents and (g) total chlorophyll contents were measured before and after a 10-day salt treatment. DW, dry weight. Data are presented as means of three biological replicates, and error bars represent SD. Asterisks indicate a significant difference between the wild-type and each transgenic plant at *P < 0.05; **P < 0.01; and ***P < 0.001 by Student’s t-test.
Overexpression of Osa-miR393a affects plant uptake of sodium and potassium

To investigate how Osa-miR393a transgenic plants perform in Na\(^+\) and K\(^+\) uptake compared to the wild-type controls, we measured root and shoot Na\(^+\) and K\(^+\) relative contents in plants grown under normal conditions and subjected to 200 mM NaCl exposure. As shown in Figure 3a and b, no significant difference in root and shoot Na\(^+\) contents was observed between Osa-miR393a transgenic and wild-type plants under normal growth conditions. When exposed to salinity stress, Na\(^+\) accumulation in shoots and roots were increased significantly in both control and Osa-miR393a plants (Figure 3a,b). More specifically, the transgenic and wild-type plants had similar Na\(^+\) accumulation in roots (Figure 3a); however, Na\(^+\) accumulation in shoots was significantly higher in Osa-miR393a transgenics than in wild-type controls (Figure 3b). Interestingly, Osa-miR393a plants accumulate more K\(^+\) than wild-type controls in both roots and shoots under both normal and salinity conditions (Figure 3c,d). Upon salt treatment, although shoot and root K\(^+\) levels started to decline in both the Osa-miR393a transgenics and wild-type plants, the decline in wild-type controls is more pronounced than in transgenic plants (Figure 3c,d). Under normal growth conditions, transgenic roots had significantly higher K\(^+\) : Na\(^+\) ratio than wild-type controls due to its higher K\(^+\) levels (Figure 3e). Upon salt treatment, however, no significant difference in K\(^+\) : Na\(^+\) ratios in shoots and roots was detected between transgenics and wild-type controls mostly due to the significantly acute decline of K\(^+\) in both genotypes (Figure 3f).

Overexpression of miR393 improves drought tolerance in transgenic plants that is associated with reduced stomata density and denser cuticle

To study miR393 function under drought stress, mature wild-type and Osa-miR393a transgenic plants grown under optimal conditions were subjected to water withholding. Serious tissue damage was observed in wild-type controls 15 days after water withholding, whereas most Osa-miR393a transgenics remained turgid (Figure 4b). Plants were then allowed to recover by sufficient watering for 6 days. Osa-miR393a transgenics were recovered from the drought-elicited damage, whereas almost all the wild-type controls died (Figure 4c). Measurement of plant RWC and EL revealed that water loss and drought-elicited cell membrane damage in Osa-miR393a plants were significantly less than that in wild-type controls (Figure 4d,e).

Further analysis showed that leaves and stems of transgenic plants were longer than wild-type controls when subjected to limited water supply treatment (Figure 5b,d). Although the number of Osa-miR393a transgenic tiller was fewer than that of the control plants under both normal and stressed conditions, there is no significant difference in shoot biomass under normal conditions, and the shoot biomass in Osa-miR393a plants was significantly increased in comparison with wild-type controls when subjected to drought stress (Figure 5a–c,g,h). Under
normal growth conditions, although transgenics have significantly fewer roots than wild-type controls, there is no significant difference in root biomass between control and transgenic plants under limited water supply (Figure 5a,b). To study whether overexpression of miR393 impacts leaf surface structure in creeping bentgrass, we examined leaf stomata density and the cuticle properties of wax crystals on leaf surface with SEM in both wild-type and Osa-miR393a transgenic plants. We found that the stomata density in transgenic plants is significantly decreased compared with wild-type controls (Figure 6a,b). Moreover, Osa-miR393a plants displayed denser cuticle and lower water loss rate than wild-type controls (Figure 6c,d). The results suggest that overexpression of miR393 improves drought tolerance in creeping bentgrass, which is associated with reduced stomata density and denser cuticle.

**Overexpression of miR393 improves heat tolerance in transgenic plants that is associated with enhanced expression of small heat-shock protein genes**

Considering that miR393 was induced under heat stress, we examined whether overexpression of miR393 alters plant response to high temperature. To this end, well-developed wild-type and Osa-miR393a plants grown under optimal conditions (day 25 °C/night 17 °C) were subjected to heat treatment (day 40 °C/night 35 °C) (Figures 7 and S5). As shown in Figure 7a,b, 13 days of heat stress lead to severe leaf damage in wild-type plants in comparison with three transgenic lines. Leaf RWC was significantly higher in transgenics than in wild-type controls after 13 days of heat stress (Park et al., 2017; Sun et al., 2016). Quantitative RT-PCR shows that AsHSP17.0 and AsHSP26.7a are induced during heat stress in both transgenic plants and controls, but the expression levels are higher in transgenics than in wild-type controls (Figure 7f,g). The result suggests that miR393 might participate in the regulation of HSP gene expression, therefore contributing to plant protection against heat stress.

Figure 4 Responses of wild-type (WT) and transgenic (TG) plants to drought stress. (a) Wild-type controls and two transgenic lines initiated from individual tillers were fully developed in pure sand in Dillen pots for 10 weeks under normal conditions in growth room before water withholding treatment. (b) The performance of wild-type plants and two independent transgenic lines 15 days after water withholding. (c) The performance of wild-type plants and two independent transgenic lines 6 days after recovery. (d) Leaf relative water content (RWC) of wild-type and transgenic plants 15 days after water withholding. (e) Leaf electrolyte leakage (EL) of wild-type and transgenic plants 15 days after water withholding. Data are presented as means of three biological replicates, and error bars represent SD. Asterisks indicate a significant difference between the wild-type and each transgenic line at *P < 0.05; **P < 0.01; and ***P < 0.001 by Student’s t-test.

© 2018 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 17, 233-251
Osa-miR393a putative target identification and their responses to stresses and auxin

In rice, miR393 targets the auxin receptors, OsAFB2 and OsTIR1. To study miR393 targets in creeping bentgrass, OsAFB2 and OsTIR1 homologs, AsAFB2 and AsTIR1 were identified through blasting OsAFB2 and OsTIR1 against the transcriptome of creeping bentgrass generated via deep sequencing (Figure 8a, Table S2, Figures S7 and S8). Interestingly, there are two putative orthologs of rice OsAFB2, AsAFB2-1 and AsAFB2-2, in creeping bentgrass genome. AsAFB2-1 and AsAFB2-2 share high identity in their amino acid sequences (Figure S8). The transcripts of AsAFB2 and AsTIR1 in Osa-miR393a transgenic lines and wild-type controls were measured by quantitative RT–PCR analysis. We observed a reduction in the transcript levels of AsAFB2 and AsTIR1 in three transgenic lines in comparison with wild-type controls (Figure 8b,c), indicating that AsAFB2 and AsTIR1 are negatively regulated by miR393 and are putative targets of miR393 in creeping bentgrass.

To determine whether miR393 targets respond to abiotic stress and auxin, quantitative RT–PCR was performed with wild-type creeping bentgrass total RNA isolated under salt, drought, heat stress and IAA treatment in leaves at various time points (0, 1.5, 3 and 6 h). The expression of AsTIR1 increased under salt, drought stresses and IAA treatment but decreased during heat treatment (Figures 9a–c and S12). Expression levels of AsAFB2 were induced when subjected to salt, drought and heat stresses, but decreased under IAA treatment (Figures 9a–c and S12). The observation indicates that miR393 responds to abiotic stress and auxin through regulating its targets, AsTIR1 and AsAFB2 in creeping bentgrass.

Overexpression of Osa-miR393a results in transcriptome changes in plant growth, development and stress regulations

miRNAs are known as master regulators in plant growth and development and response to plant stresses via coordinating multiple stress-responsive factors. Thus, besides the direct targets...
of miR393, other plant growth and stress-related elements might be regulated by miR393. To detect miR393-mediated transcripts that may be involved in plant growth and development, a comparative transcriptome analysis was conducted using leaf samples of Osa-miR393a transgenic line (TG-4) and wild-type (WT) controls. Under the criteria of false discovery rate <0.05 and log2 fold change ≥1, a total of 8871 unigenes were differentially expressed in Osa-miR393a transgenics compared with WT controls, of which 5319 (60%) were up-regulated, whereas 3552 (40%) down-regulated (Figure S9). Functional annotation of putative gene products indicated that overexpression of Osa-miR393a affected multiple biological processes including cellular response to stimulus, developmental, biological regulation, transporter activity, molecular function regulator antioxidant response to stimulus, developmental, biological regulation, miR393a affected multiple biological processes including cellular response to stimulus, developmental, biological regulation, transporter activity, molecular function regulator antioxidant response.

Discussion

MiR393-TIR1/AFB2 module plays an important role in regulating creeping bentgrass development and abiotic stress resistance

miR393 has previously been identified in a variety of plant species including monocots and dicots (Bian et al., 2012; Jagadeeswaran et al., 2009; Jones-Rhoades and Bartel, 2004; Navarro and Jones, 2006; Xia et al., 2012; Zhang et al., 2009). In this study, the stem-loop RT-qPCR analysis demonstrated that creeping bentgrass miR393 was strongly expressed in stems and leaves, but lowly expressed in roots and flowers (Figure S1a). Previous studies indicated that miR393 regulates auxin signalling pathway by
Figure 7  Responses of wild-type (WT) and transgenic (TG) plants to heat stress. Wild-type controls and three transgenic lines were fully developed in containers for 10 weeks under normal conditions in growth room. Plants were then transferred to the growth chamber and subjected to heat stress at 40 °C in the light and 35 °C in the dark for 13 days. The relative humidity in the chamber was 60%–80%. Plant tissues were harvested for further analysis after heat treatment. (a) Wild-type controls and three transgenic lines initiated from the same number of tillers were trimmed to the same height before the heat stress test. (b) Performance of wild-type and transgenic plants subjected to heat stress at 40 °C in the light and 35 °C in the dark for 13 days. (c) Relative water contents (RWC), (d) electrolyte leakage (EL) values and (e) total chlorophyll contents were measured before and after 13-day heat treatment. (f) Expression profiles of AsHSP17.0 in wild-type and transgenic leaf tissues under 40 °C (0–3 h). (g) Expression profiles of AsHSP26.7a in wild-type and transgenic leaf tissues under 40 °C (0–3 h). DW, dry weight. Data are presented as means of three biological replicates, and error bars represent SD. Asterisks indicate a significant difference between the wild-type and each transgenic plant at ***P < 0.001 by Student’s t-test.

Figure 8  Putative miR393 target identification in creeping bentgrass. (a) Comparison of target sites in the three putative miR393 target genes in creeping bentgrass with the mature sequence of Osa-miR393. Expression levels of AsTIR1 (b) and AsAFB2 (c) in wild-type plants (WT) and three transgenic (TG) lines examined via RT–qPCR. Data are presented as means of three biological replicates, and error bars represent SD. Asterisks indicate a significant difference of expression levels between the wild-type and each transgenic line at ***P < 0.001 by Student’s t-test.
tion. Tiller number is a very important turf trait and a key factor unexpectedly, thus preventing us from its further characteriza-

lines TG3 and TG5 also exhibited an early senescence and died

promoted by low concentrations of auxin to a maximum, but

depending on their concentrations. For example, plant growth is

way analogous to the two-folded responses plant hormones elicit

miR393 might play a delicate and interesting role in regulating its

(Figures 1a-c,i-k and S2c). We speculate that the dose effect of

displayed fewer and longer tillers than WT controls

Osa-miR393a had shorter tillers than WT control plants, resulting

in dwarf phenotype (Figures S2c and S3a), whereas the other

transgenic lines with relatively low expression levels of

transgenic creeping bentgrass plants, suggesting that the regulation module of

miR393 in the auxin pathway is conserved in different plant

species. Overexpressing miR393 in transgenic rice caused reduced

determination of turf density. As shown in Figure S3, WT controls and

transgenic plants were initiated from the same amount of tillers and

maintained in a big pot allowing them to grow closely from each other and become quite dense, similar to that of the field-grown turf. Under such conditions, transgenic plants display reduced growth. In particular, TG1 plants exhibit a semi-dwarf-like phenotype associated with a higher accumulation of the mature miR393 than TG2 and TG4. However, the transgenic plants exhibit longer internodes than WT controls initiated from a single tiller (Figure 1k). Note that under such growth conditions, the semi-dwarf like phenotype observed in TG1 densely grown in a big pot (Figure S3) was not quite obvious (Figure 1a). This may be related to the less growth competition for each individual plant under such conditions than for the plants densely grown with more growth competition. The miR393 transgenic creeping bentgrass plants had fewer tillers than wild-type controls, which is undesirable for turf. This important piece of information offers great insights for turfgrass genetic engineering with miRNA393, suggesting that engineering-specific miRNA targets would be a better strategy in turf trait modification to avoid pleiotropic effects bestowed by the master regulator, miRNAs.

The role of miR393-TIR1/AFBs module in regulating abiotic stress response has been well characterized in some plant species. In Arabidopsis, transgenic plants overexpressing miR393 displayed decreased susceptibility to auxin and enhanced plant sensitivity to salt and osmotic stress through suppressing target genes (Chen et al., 2011, 2015b). Similarly, overexpression of miR393 also led to decreased sensitivity to auxin and conferred reduced tolerance to salt and drought in transgenic rice due to the down-regulation of its target mRNAs (Xia et al., 2012). However, unlike Arabidopsis and rice, transgenic tobacco

Figure 9 Expression patterns of the two putative miR393 targets of creeping bentgrass under salt, drought and heat stress conditions by real-time RT–PCR analysis. (a) Real-time RT–PCR analysis of AsTIR1 and AsAFB2 gene expression in wild-type leaf tissues after exposure to 200 mM NaCl solution (0–6 h). (b) Real-time RT–PCR analysis of AsTIR1 and AsAFB2 gene expression in wild-type leaf tissues after exposure to air dry (0–6 h). (c) Real-time RT–PCR analysis of AsTIR1 and AsAFB2 gene expression in wild-type leaf tissues after exposure to heat stress at 40 °C (0–6 h). Data are presented as means of three biological replicates, and error bars represent SD. Asterisks indicate significant differences of gene expression levels between untreated and stress-treated leaf tissues: **P < 0.01; and ***P < 0.001 by Student’s t-test.

Expression patterns of the two putative miR393 targets of creeping bentgrass under salt, drought and heat stress conditions by real-time RT–PCR analysis. (a) Real-time RT–PCR analysis of AsTIR1 and AsAFB2 gene expression in wild-type leaf tissues after exposure to 200 mM NaCl solution (0–6 h). (b) Real-time RT–PCR analysis of AsTIR1 and AsAFB2 gene expression in wild-type leaf tissues after exposure to air dry (0–6 h). (c) Real-time RT–PCR analysis of AsTIR1 and AsAFB2 gene expression in wild-type leaf tissues after exposure to heat stress at 40 °C (0–6 h). Data are presented as means of three biological replicates, and error bars represent SD. Asterisks indicate significant differences of gene expression levels between untreated and stress-treated leaf tissues: **P < 0.01; and ***P < 0.001 by Student’s t-test.
overexpressing an Arabidopsis miR393 gene exhibited altered auxin sensitivity and enhanced salt tolerance due to auxin signal suppression via negative regulation of target NITIR1 (Feng et al., 2010). Similarly, our results clearly demonstrate that transgenic creeping bentgrass overexpressing Osa-miR393a gene had enhanced drought and salt stress tolerance in comparison with wild-type controls (Figures 2, 4 and 5). Our data together with previous research suggest that the mechanisms in which miR393-regulated downstream pathways (Chen et al., 2015b). In our study, AsTIR1 and AsAFB2 expressions are induced by many abiotic stresses, such as salt, dehydration and heat (Figure 9), suggesting that the putative miR393 target genes are directly involved in miR393-mediated plant responses to abiotic stress. Additionally, our RNA-seq data show that several stress-responsive transcription factors were also up- or down-regulated in Osa-miR393a transgenic plants (Table 1). Thus, we speculate that miR393 may serve as a master regulator to cope with different environmental stresses through integrating various regulatory pathways in creeping bentgrass.

**Morphological change and drought stress tolerance in Osa-miR393a transgenics**

Plants can cope with water deficit through morphological changes in root and leaf (Fang and Xiong, 2015). Leaves are not only very important in plant photosynthesis and respiration, but also considered to be vital in regulating stress tolerance (Atkin et al., 2000; Nautiyal et al., 2008, Terry and Ulrich, 1974). In our study, Osa-miR393a transgenic plants exhibit decreased stomata density, denser cuticle on leaf surface (Figure 6a,b,d) and wider

### Table 1

| Gene ID Functional annotation log2 fold (TG/WT) |
|-----------------------------------------------|
| Plant development-related genes |
| DN224490_c0_g1_i1 65-kDa microtubule-associated protein 1-like 2.20 |
| DN238591_c1_g1_i2 65-kDa microtubule-associated protein 5-like −4.80 |
| DN234899_c3_g2_i2 65-kDa microtubule-associated protein 6-like 1.84 |
| DN230175_c0_g1_i2 COBRA-like protein 1.32 |
| DN218283_c2_g5_i3 Cyclin-P1-like −4.46 |
| DN220973_c4_g1_i4 Cyclin-P3-like −2.93 |
| DN223637_c0_g2_i3 Cyclin-P4-like −10.41 |
| DN226448_c2_g7_i1 Cyclin-P4-like −9.60 |
| DN220853_c0_g1_i3 Auxin-responsive protein SAUR71 3.74 |
| DN236012_c1_g13_i1 Auxin-responsive protein SAUR36 2.33 |
| DN212321_c0_g1_i3 Epidermal patterning factor-like protein 2.42 |
| DN223880_c2_g3_i4 Cytokinin dehydrogenase 11 −2.19 |
| DN236376_c0_g1_i2 Cytokinin dehydrogenase 9 −1.91 |
| Transcription factors |
| DN201921_c0_g3_i1 WRKY transcription factor 53 2.88 |
| DN219285_c3_g2_i3 WRKY transcription factor 48 3.34 |
| DN222735_c2_g1_i2 WRKY transcription factor 12 2.24 |
| DN223738_c3_g2_i1 WRKY transcription factor 54 −6.88 |
| DN230932_c5_g11_i3 WRKY transcription factor 72 −2.29 |
| DN214957_c5_g5_i2 Transcription factor TCP8 2.29 |
| DN216241_c0_g3_i2 Transcription factor TCP21 2.51 |
| DN223851_c1_g2_i1 Transcription factor TCP2 2.03 |
| DN233603_c3_g7_i2 Transcription factor TCP7 2.46 |
| DN204421_c0_g5_i1 Transcription factor, MADS-box −6.50 |
| DN226502_c1_g1_i1 Transcription factor, MADS-box 2.22 |
| DN207922_c0_g2_i3 Transcription factor MYC/MYB 1.76 |
| DN224773_c1_g1_i4 Transcription factor MYC/MYB 2.12 |
| DN216428_c0_g2_i2 Dehydration-responsive element-binding protein 6.53 |
| Potassium transport-related genes |
| DN223158_c1_g1_i1 Voltage-gated potassium channel activity 7.31 |
| DN232008_c2_g5_i2 Voltage-gated potassium channel subunit beta 1.90 |
| DN232008_c2_g18_i1 Voltage-gated potassium channel subunit beta 2.33 |
leaves (Figure 1f,g). As is known, the rate of transpiration can be regulated by stomata density and stomata opening (Mehri et al., 2009). Thus, decreased stomata density on leaf surface had a significant positive correlation with plant drought stress tolerance due to a reduced transpiration rate (Hepworth et al., 2015; Luo et al., 2013). As for leaf surfaces, they are covered with a cuticular wax layer that is the first barrier to protect plants against the environmental stimulation. They can effectively reduce water transpiration loss and enhance plant resistance to drought stress (Cameron et al., 2006, 2006; Islam et al., 2009; Zhang et al., 2005). Hence, the phenotype of denser cuticles on transgenic leaf surface is considered as a positive trait in regulating plant drought stress tolerance. However, wider leaves are actually considered as a negative trait in regulating drought stress tolerance due to increased transpiration area (Craw et al., 2004; Déák et al., 2011). Conclusively, whether the plant cultivars are more resistant to drought stress or not is dependent on the balance of trade-offs of several morphological traits and other factors. This is similar to the case of miR319, in which other characteristics of leaves, such as stomatal aperture, stomatal density, increased leaf thickness and increased wax content on leaf surface, may compensate the water loss by wider leaves (Zhou et al., 2013). To further explore what caused the morphological change in Osa-miR319 plants, we examined the RNA-seq data backed up by qRT–PCR confirmation and found that the expression of AsEPF, a homolog of Arabidopsis EPIDERMAL PATTERNING FACTOR (EPF)-like gene was significantly higher in transgenics under normal conditions than that in WT controls (Figure 10h).

Figure 10  Expression levels of genes selected from RNA-seq data in wild-type (WT) and transgenic (TG) plants examined via RT–qPCR. Data are presented as means of three biological replicates, and error bars represent SD. Asterisks indicate a significant difference of expression levels between the wild-type and the transgenic plants: *P < 0.001 by Student’s t-test.
Thus, further exploration about how miR393 functions to regulate AsEPF expression to control stomata density change will provide information for a better understanding of molecular mechanisms of miR393-mediated drought tolerance in transgenic creeping bentgrass.

Mechanisms of high-salinity resistance in Osa-miR393a transgenic plant

To further explore the underlying mechanisms of the enhanced salt stress tolerance in Osa-miR393a transgenic plants, we analysed Na\(^+\) and K\(^+\) uptake in both Osa-miR393a transgenic and wild-type control plants. As shown in Figure 3b,e, Osa-miR393a transgenic plants perform better than wild-type controls in maintaining K\(^+\) levels in shoots and roots under both the normal and stressed conditions. This phenotype correlates well with our RNA-seq and qRT-PCR data that the expression of AsNHX1 is significantly up-regulated in transgenic plants (Figure S11a). Considering that NHX1 and NHX2 play important roles in controlling potassium uptake into vacuoles to mediate cell turgor and stomata functions in Arabidopsis (Barragán et al., 2012), these results together indicate that the impact of miR393 on potassium uptake and transport is significant. However, as for Na\(^+\) accumulation shown in Figure 4a,d, miR393 has no impact on roots. Interestingly, when exposed to 200 mM NaCl, Osa-miR393a transgenic plants accumulate more sodium than wild-type controls (Figure 3d). These data suggest that Osa-miR393a transgenic plants do not adopt a salt exclusion mechanism to reduce sodium toxicity like what Osa-miR319a plants do (Zhou et al., 2013). Instead, they might gain more salt tolerance through sodium sequestration into vacuoles. Thus, the increased accumulation of sodium in Osa-miR393a plants could lead to decreased cell osmotic potential, thus facilitating plant water uptake and leaf turgor maintenance under salinity conditions, consistent with the observation in transgenic creeping bentgrass overexpressing an Arabidopsis vacuolar H\(^+\)-pyrophosphatase (AVP1) gene (Li et al., 2010). Indeed, the expression of AVP1 is significantly up-regulated in Osa-miR393a transgenic plants (Figure S11b). Although our data demonstrate the correlation between miR393 and the control of vacuolar dynamics, it remains unclear how miR393 functions in this process. It would be very interesting to dissect the role miR393 plays in determining ion exchange in creeping bentgrass by regulating AVP1 and AsNHX1 in the future.

miR393 positively regulates plant heat stress response in creeping bentgrass

Previous studies in various plant species suggested that miR393 plays a crucial role in response to multiple environmental cues, such as drought, salt, alkali, cold and aluminium stress (Bai et al., 2017; Gao et al., 2011; Liu et al., 2017; Xia et al., 2012; Xie et al., 2007; Zhou et al., 2008). However, there has been no report about the possible involvement of miR393 in plant response to heat stress so far. In this study, we show that under high temperature, Osa-miR393a transgenic plants remained green with only minor heat damage and slightly reduced biomass compared to wild-type controls, suggesting an enhanced heat tolerance in Osa-miR393a transgenic plants (Figure 7). It is worth noting that TG1 seems to suffer a little more heat damage than other transgenic lines despite its enhanced heat tolerance compared to wild-type controls (Figure S5). This might be associated with the higher expression of miR393 in this transgenic line than in the other two lines (TG2 and TG4). Interestingly, this minor difference in heat tolerance does not seem to be manifested when testing plant heat response under densely grown conditions. Presumably, the densely grown plants might be more advantageous than individually grown plants in buffering heat-elicited damage. In Chinese cabbages, the high content of cuticular wax in heat-tolerant cultivar plays an important role in affecting water balance in plants under heat stress, suggesting that the effects of heat stress are likely involved in drought stress under high-temperature conditions (Kuo et al., 1988). Our research shows that Osa-miR393a transgenic plants exhibit denser cuticle than WT controls under normal conditions (Figure 6d), which also implies higher wax content in Osa-miR393a transgenic plants. Thus, we speculate that the enhanced heat stress tolerance in transgenic creeping bentgrass is associated with increased cuticular wax coverage, which can positively regulate water balance in plants under high temperature.

The heat-shock proteins (HSPs), including small heat-shock proteins (sHSPs), accumulate in response to heat stress. They have a chaperone activity for a protective effect at high temperature to prevent protein misfolding and aggregation (Vierling, 2003; Wang et al., 2004). Heat tolerance is associated with induced expression of heat-shock proteins in plants (Queitsch et al., 2000; Sun et al., 2002; Zhong et al., 2013). Overexpression of HSP leads to enhanced heat tolerance has also been observed in various plant species (Kim et al., 2012; Merino and Gómez, 2014; Mu et al., 2013; Murakami et al., 2004; Sato and Yokoya, 2008; Sun et al., 2012). In creeping bentgrass, chloroplast-localized small heat-shock proteins (CP-sHSPs) play an important role in heat tolerance (Wang and Luthe, 2003). It has previously been demonstrated that transgenic creeping bentgrass overexpressing OsS21, a rice gene encoding SUMO E3 ligase, exhibit enhanced heat tolerance, and this enhancement is correlated with significantly induced expression of ApHSP16.5 (renamed as AsHSP17 in this study) and AsHSP26.7a compared to wild-type control plants (Li et al., 2013). In this study, when subjected to heat stress, AsHSP17 and AsHSP26.7a genes were all induced in both wild-type and Osa-miR393a transgenic plants, but the induced expression of AsHSP17 and AsHSP26.7a in Osa-miR393a transgenic plants was significantly more pronounced than in wild-type controls either at all the time (AsHSP17) or 0.5 h after exposure to heat stress (AsHSP26.7a) (Figure 7f,g). This result suggests that miR393 may be involved in heat stress tolerance in creeping bentgrass via positive regulation of certain CP-sHSPs in heat response pathways. Additionally, down-regulated expression of the putative miR393 target genes, AsTIR1 and AsAFB2 in response to heat stress (Figure 9c), suggests their possible involvement in determining plant capacity of coping with heat stress. Further studies will provide information for a better understanding of the molecular mechanisms of miR393-mediated plant resistance to heat stress through overexpression and knockout of miR393 target genes in creeping bentgrass.

In summary, miR393 is induced during salinity, heat, drought stress and auxin treatment. miR393 mediates plant abiotic stress responses through directly repressing the expression of its targets AsTIR1 and AsAFB2. In addition, miR393 positively or negatively regulates sHSPs (AsHSP17.0 and AsHSP26.7a), certain transcription factors, ion transport-related genes (AsVP1 and AsNHX1) and AsEFP, which leads to the enhanced tolerance to salinity, heat and drought stress. Taken together, our data suggest that miR393 forms a regulatory network to integrate various signals in plant response to abiotic stress (Figure 11). Our data also demonstrate a striking difference in miR393-mediated plant
development and stress responses between various species (Liu et al., 2017; Xia et al., 2012), highlighting the significance of the species-dependent miR393 regulatory networks. The phenotype difference between species in miR393-mediated plant development and stress responses might result from the evolution of miRNA regulatory programs. In some plant species, conserved miRNAs may have gained unique targets over time, or alternatively, conserved miRNA-target gene modules could have gained new functions during evolution (Willmann and Poethig, 2007). It is therefore conceivable that creeping bentgrass miR393 might gain new target and/or new miR393-target signalling pathway, attributable to the characteristic phenotypes, such as reduced tillers and enhanced stress tolerance when overexpressing miR393 in transgenics (Figure 1). In fact, the phenomenon that conserved miRNAs play opposite roles in different plant species has previously been observed. For instance, constitutive expression of miR169 enhanced drought tolerance in transgenic tomato, but resulted in increased sensitivity to drought stress in tobacco plants. For example, conserved miRNAs play opposite roles in different plant species (Liu et al., 2017; Xia et al., 2012), highlighting the significance of the species-dependent miR393 regulatory networks. The phenotype difference between species in miR393-mediated plant development and stress responses might result from the evolution of miRNA regulatory programs. In some plant species, conserved miRNAs may have gained unique targets over time, or alternatively, conserved miRNA-target gene modules could have gained new functions during evolution (Willmann and Poethig, 2007). It is therefore conceivable that creeping bentgrass miR393 might gain new target and/or new miR393-target signalling pathway, attributable to the characteristic phenotypes, such as reduced tillers and enhanced stress tolerance when overexpressing miR393 in transgenics (Figure 1). In fact, the phenomenon that conserved miRNAs play opposite roles in different plant species has previously been observed. For instance, constitutive expression of miR169 enhanced drought tolerance in transgenic tomato, but resulted in increased sensitivity to drought stress in transgenic Arabidopsis (Li et al., 2008; Zhang et al., 2011). Besides, the differences in plant development (such as tiller numbers) between transgenics of various plant species overexpressing miR393 might also result from varying sensitivities to hormone auxin. It is known that higher concentration of auxin promotes tiller elongation and inhibits the growth of lateral buds (apical dominance), whereas lower concentration of auxin promotes the growth of lateral tillers. Although it has been shown that miR393 overexpression leads to hyposensitivity to auxin (Chen et al., 2011; Liu et al., 2017; Xia et al., 2012), this sensitivity to auxin could vary among plant species, or even plant tissues. Without the knowledge about auxin concentration for optimal plant growth in a given species, it is hard to determine whether the hyposensitivity leads to the maintenance or removal of the apical dominance. Therefore, different plant species may exhibit different phenotypes under similar auxin concentration. As the upstream regulators, miRNAs not only directly affect their targets but also indirectly impact other non-target genes. Thus, besides the direct miR393 targets, plant development and stress responses may also depend on other downstream functional genes (indirect miR393 targets), which could vary in different species, and therefore contribute differently to plant development and stress responses. Information about the species-associated differences in miR393-mediated plant development and stress responses greatly enriches our knowledge and understanding about the biological functions of miR393. The data obtained in a perennial crop species provide critical information for the development of new biotechnology approaches for genetically engineering other important crops for enhanced agricultural production.

**Experimental procedures**

Cloning of the *Osa-miR393a* gene and construction of overexpression vector

To make the *Osa-miR393a* overexpression construct, the full-length rice (*Oryza sativa* *Osa-miR393a* cDNA fragment containing a stem-loop structure was amplified by PCR using the forward and reverse primer set and cloned into the binary vector pZH01 (Xiao et al., 2003), under the CaMV35S promoter and the CaMV35S promoter driving the *hgy* gene for hygromycin resistance as a selectable marker. The construct containing the overexpression cassette for *Osa-miR393a* was transformed into the *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Table S1 has summarized all the primers used in this study.

Plant materials and transformation

The transformed *Agrobacterium* containing the overexpression construct of *Osa-miR393a* was used to infect the embryonic callus induced from the mature seeds of creeping bentgrass (*A. stolonifera* L.) cv. Penn A-4 (Supplied by HybriGene, Hubbard, OR, USA) following the previous protocol (Luo et al., 2004).

Plant propagation, maintenance and abiotic stress treatments

The newly generated transgenic creeping bentgrass plants overexpressing *Osa-miR393a* and wild-type controls were subject to clonal propagation by multiplying their tillers. All the plants were maintained as described previously (Yuan et al., 2015; Zhou et al., 2013).

To investigate the plant response to salt stress, plants grown in cone-tainers and small-sized pots were immersed in NaCl solution supplemented with 0.2 g/L water-soluble fertilizer (20 : 10 : 20 nitrogen : phosphorus : potassium; Peat-Lite...
MiR393 regulates development and stress responses

Special; Scotts). Based on literatures and our previous work, a concentration of 200 mM NaCl is adequate for salt stress test in creeping bentgrass. Considering that plant materials for salt test were grown in pure silicon sand, which contains water, we decided to use an increased NaCl concentration of 250 mM for plant salt treatment. Shoots were harvested 10 days after salt treatments for further physiological analysis. Replicates of the salt-treated plants were watered with 0.2 g/L water-soluble fertilizer every other day for recovery from salt stress and documented by photography. Wild-type leaves were collected at 0, 1.5, 3 and 6 h after salt stress treatment for analysis of miR393 expression.

To evaluate heat stress tolerance, plants grown in cone-tainers and Elite 1200 pots, respectively, were immersed in 0.2 g/L water-soluble fertilizer in a growth chamber (Conviron, Controlled Environments Inc., Pembroina, ND) and subjected to heat stress by heating the plants to 40 °C in the light and 35 °C in the dark for 14 days. The relative humidity in the chamber was 60%–80%, and the heat-stressed plants were well-watered every 2 days with distilled water. wild-type leaves were collected at 0, 1.5, 3 and 6 h after heat stress treatment for analysis of miR393 expression.

To study plant response to drought stress, two events of TG (TG1 and TG4) and WT control plants were vegetatively propagated from stolons or a single tiller of the same size (limited water supply) as previously described (Li et al., 2013; Zhou et al., 2013). The plants in the big-sized pots (10 cm × 10.5 cm; Dillen Products, Middlefield, OH) or cone-tainers with pure sand were maintained under normal conditions in growth room for 10 weeks and then subjected to drought stress by water withholding or limited water supply (10 mL for each plant every 2 days). For miR393 expression analysis, wild-type plants taken out of the pure sand were dried with a paper towel and then laid on the bench for air drying. The leaf samples were collected at 0, 1.5, 3 and 6 h after drought treatment. Wild-type leaves were collected at 0, 1.5, 3 and 6 h after drought treatment. Wild-type leaves were collected at 0, 1.5, 3 and 6 h after drought treatment. Wild-type leaves were collected at 0, 1.5, 3 and 6 h after drought treatment. Wild-type leaves were collected at 0, 1.5, 3 and 6 h after drought treatment. Wild-type leaves were collected at 0, 1.5, 3 and 6 h after drought treatment. Wild-type leaves were collected at 0, 1.5, 3 and 6 h after drought treatment. Wild-type leaves were collected at 0, 1.5, 3 and 6 h after drought treatment.

Identification of transgenic plants, isolation of plant RNA and gene expression analysis

Genomic DNA was isolated from the putative transgenic and wild-type plants. The PCR was used to screen for putative transgenic plants with the primers Hyg-F and Hyg-R (Table S1).

Total RNA was extracted from plant leaf with the Trizol reagent and treated with RNase-free DNase (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using SuperScript III Reverse Transcriptase Kit (Invitrogen) following the manufacturer’s protocol. AsUBQ was used as the internal reference for assessing gene expression level in creeping bentgrass. Semi-quantitative RT–PCR was conducted using the following program: 5-min denaturation at 94 °C, followed by 24 to 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and completed with an extension step of 5 min at 72 °C. Real-time RT–PCR was conducted in a total volume of 25 μL containing 12.5 μL of IQ SYBR-Green Supermix (Bio-Rad Laboratories) on the Bio-RadQ5 real-time detection system according to the manufacturer’s instructions using the following program: 5 min denaturation at 94 °C, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The measurements were obtained using the relative quantification method (Livak and Schmittgen, 2012). The primers used were listed in Table S1. Stem-loop RT–qPCR was performed according to the protocol of Varkonyi-Gasic et al. (2007).

Measurement of mineral content, leaf RWC, EL, chlorophyll and MDA content

To examine Na+ and K+ contents, plants were grown hydroponically to facilitate root sampling. Under this condition, plant roots were in direct contact with NaCl solution, we therefore used 200 mM, a lower concentration of NaCl than that for plants grown in sands, for salt treatment. The leaves and roots of the wild-type and transgenic plants were collected before and after a 10-day treatment of 200 mM NaCl. A total of 0.5 g dried materials was used in each sample. The whole procedure of determination of the mineral contents in plants was described in previous protocols (Haynes, 1980; Li et al., 2010).

Plant leaf RWC, EL, chlorophyll content and MDA content were measured following previous protocols (Bates et al., 1973; Dhindsa et al., 1981; Li et al., 2010).

Measurement of water loss rate

To detect the water loss rate under dehydration conditions, transgenic plants and wild-type plants were exposed to air at room temperature and weighed at the designated times.

Plant histological analysis

Histological analysis for the leaf and stem cross sections was performed as described previously (Yuan et al., 2015). In this study, sections were stained using Toliudine blue. For leaf cuticle features observation, leaves of the same age at the same relative positions sampled from the longest tiller of the wild-type and transgenic plants were fixed in 2.5% (vol/vol) glutaraldehyde. Samples were sputter-coated with gold particles and coated surfaces were viewed using a JEOL JSM-6390LV scanning electron microscope.

Measurements of leaf stomatal density

For stomatal density measurement, leaves of the same age at the same relative positions were sampled from the longest tiller of the wild-type and transgenic plants. A drop of clear nail polish was applied to the leaf upper epidermis and let it dry completely. The films were then peeled off and observed under a light microscope (MEJI EMZ-STR).

cDNA library preparation and Illumina sequencing, differential expression and GO enrichment analyses

Total RNA was isolated from leaves of wild-type and Osamir393a TG4 plants grown under normal conditions with Trizol reagent (Invitrogen). Poly (A)-containing mRNA molecules were purified from total RNA using poly (T) oligo-attached magnetic beads and then fragmented into 150- to 250-bp pieces using fragmentation reagent, and the first-strand cDNA was generated using random hexamer-primed reverse transcription. The second-strand cDNA was generated upon completion of the first-strand synthesis with dTTP being replaced by dUTP. The synthesized cDNA was subsequently subjected to end-repair followed by 3’ adenylation. Adaptors were ligated to the ends of these 3’-adenylated cDNA fragments, and the second-strand cDNA degradation was performed by UDG (uracil–DNA glycosylase). The products of the ligation reaction were purified on TAE-agarose gel. Many rounds of PCR amplification were performed to enrich the purified cDNA template. The cDNA library was validated by the Agilent Technologies 2100 Bioanlyser and the
ABI Step One Plus Real-Time PCR System. Paired-end sequencing of each library was performed using the HiSeq 2000 (Illumina Technologies) platform following the manufacturer’s instructions. The removal of poor quality sequences and trimming of adaptor sequences from the raw sequence data were carried out using cutadapt (v1.8.1). The clean sequencing data for each sample were assembled to transcripts using Trinity software (Haas et al., 2013). All assembled transcripts from total samples were clustered by cdhit (v4.6) with default parameters (Li and Godzik, 2006). The clustered transcripts were then aligned to Rfam database (v.11) (http://www.sanger.ac.uk/Software/Rfam/) to exclude those matching rRNAs. The open-reading frame regions of transcripts were predicted by the Transdecoder (v.2.0.1). RSEM tool of transcripts were predicted by the Transdecoder (v.2.0.1). RSEM method was adopted to quantify total expressed transcripts with the TPM (Transcripts per million) value (Li and Dewey, 2011). Tool edgeR package was used to identify the differential expression genes on the read count values of genes (Nikolayeva and Robinson, 2014). The fold change between the two groups was calculated as: logFC = log2 (transgenic group/wild group). Gene expression was measured using a logFC > 1 and a q value < 0.05 (it depends), was defined as differential expression genes in this study. The ‘blastp’ program (v.2.2.29) was used to annotate these expressed transcripts’ function with NR, NOG and swissport database with the cutoff of e-value < 1e−5. The software of InterProScan (v.5.15-54.0) was used to annotate the domains, gene families and Gene Ontology (GO) functions. GO classification was used to obtain GO functional class for all the transcripts.

Acknowledgements

This project was supported by Biotechnology Risk Assessment Grant Program competitive grant no. 2010-33522-21656 from the USDA National Institute of Food, Agriculture and the USDA grant CSREES SC-1700450, United States Golf Association, Inc. and the China Scholarship Council Fellowship. This is Technical Contribution No. 6292 of the Clemson University Experiment Station.

Conflict of interest

The authors declare no conflict of interests.

References

Agarwal, P.K., Agarwal, P., Reddy, M.K. and Sopory, S.K. (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep. 25, 1263–1274.

Aguilera-Martínez, J.A. and Sinha, N. (2013) Analysis of the role of Arabidopsis class I TCP genes. AITCP1, AITCP8, AITCP22, and AITCP23 in leaf development. Front. Plant Sci. 4, 406.

Atkin, O.K., Millar, A.H., Gardestrom, P. and Day, D.A. (2000) Photosynthesis, carbohydrate metabolism and respiration in leaves of higher plants. In Photosynthesis: Physiology and Metabolism (Leeoodg, R.C., Sharkey, T.D. and Caemmerer, S.V., eds), pp. 153–175. Dordrecht: Springer, Netherlands.

Atkinson, N.J. and Unwin, P.E. (2012) The interaction of plantiotic and abiotic stresses: from genes to the field. J. Exp. Bot. 63, 3523–3543.

Bai, B., Bian, H., Zeng, Z., Hou, N., Shi, B., Wang, J., Zhu, M. et al. (2017) miR393-mediated auxin signaling regulation is involved in root elongation inhibition in response to toxic aluminum stress in Barley. Plant Cell Physiol. 58, 426–439.

Barragán, V., Leidi, E.O., Andrés, Z., Rubio, L., Luca, A.D., Fernández, J.A., Cubero, B. et al. (2012) Ion exchangers NHX1 and NHX2 mediate active potassium uptake into vacuoles to regulate cell turgor and stomatal function in Arabidopsis. Plant Cell, 24, 1127–1142.

Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 116, 281–297.

Bary, A.E., Balej-Jeeres, J. and Weretilnyk, E. (2000) Responses to abiotic stresses. In Biochemistry and molecular biology plants (Buchan, B.B., Gruissem, W. and Jones, R.L., eds), pp. 1158–1249. New Delhi: I.K. International Publishing House.

Bates, L.S., Waldren, R.P. and Teare, I.D. (1973) Rapid determination of free proline for water-stress studies. Plant Soil, 39, 205–207.

Beaucœur, L., Yu, A. and Bouché, N. (2010) microRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNAs in Arabidopsis. Plant Mol. Biol. 62, 463–465.

Bian, H., Xie, Y., Guo, F., Han, N., Ma, S., Zeng, Z., Wang, J. et al. (2012) Distinctive expression patterns and roles of the miRNA939/TIR1 homolog module in regulating flag leaf inclination and primary and crown root growth in rice (Oryza sativa). New Physiol. 196, 149–161.

Cameron, K.D., Teeece, M.A. and Smart, L.B. (2006) Increased accumulation of cuticular wax and expression of lipid transfer protein in response to periodic drying events in leaves of tree tobacco. Plant Physiol. 140, 176–183.

Chaves, M.M., Maroco, J.P. and Pereira, J.S. (2003) Understanding plant responses to drought-from genes to the whole plant. Funct. Plant Biol. 30, 233–254.

Chen, Z.H., Bao, M.L., Sun, Y.Z., Yang, Y.J., Xu, X.H., Wang, J.H., Han, N. et al. (2011) Regulation of auxin response by miR393-targeted is involved in normal development in Arabidopsis. Plant Mol. Biol. 77, 619–629.

Chen, M.X., Lung, S.C., Du, Z.Y. and Chye, M.L. (2014) Engineering plants to tolerate abiotic stresses. Biocatal. Agric. Biotechnol. 3, 81–87.

Chen, L., Luan, Y. and Zhai, J. (2015a) Sp-miR396a-5p acts as a stress-responsive genes regulator by conferring tolerance to abiotic stresses and susceptibility to Phytophthora nicotianae in transgenic tobacco. Plant Cell Rep. 34, 2013–2025.

Chen, Z., Hu, L., Han, N., Hu, J., Yang, Y., Xiang, T., Zhang, X. et al. (2015b) Overexpression of a mR393-resistant form of transport inhibitor response protein 1 (mTIR1) enhances salt tolerance by increased osmoregulation and Na⁺ exclusion in Arabidopsis thaliana. Plant Cell Physiol. 56, 73–83.

Crockett, C.E., den Boer, B.G., Healy, J.M. and Murray, J.A. (2000) Cyclin D control of growth rate in plants. Nature, 405, 575–579.

Craw, D., Wilson, N. and Ashley, P.M. (2004) Investigation of physiological responses and leaf morphological traits of wheat genotypes with contrasting drought stress tolerance. Appl. Earth Sci. Inn. Trans. 113, B3–B10.

Deik, C., Jäger, K., Fabián, A., Nagy, A., Albert, Z., Miskó, A., Barnabás, B. et al. (2011) Investigation of physiological responses and leaf morphological traits of wheat genotypes with contrasting drought stress tolerance. Acta Biologica Szegediensis, 55, 69–71.

Dharmasiri, N., Dharmasiri, S. and Estelle, M. (2005) The F-box protein TIR1 is an auxin receptor. Nature, 435, 441–445.

Dhindsa, R.S., Plumb-Dhindsa, P. and Thorpe, T.A. (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. 32, 93–101.

Ding, Y.F. and Zhu, C. (2009) The role of microRNAs in copper and cadmium homeostasis. Biochem. Biophys. Res. Commun. 385, 6–10.

Ding, X., Cao, Y., Huang, L., Zhao, J., Xu, C., Li, X. and Wang, S. (2008) Acquisition of the indole-3-acetic acid-amido synthetase GH3-8 suppresses expansin expression and promotes salicylate- and jasmonate-independent basal immunity in rice. Plant Cell, 20, 228–240.

Fang, Y. and Xiong, L. (2015) General mechanisms of drought response and their application in drought resistance improvement in plants. Cell. Mol. Life Sci. 72, 673–689.

FAO. (1996) Food requirements and population growth. http://www.fao.org/.

Feng, X.M., You, C.X., Qiao, Y., Mao, K. and Hao, Y.J. (2010) Ecopic overexpression of Arabidopsis AtmR339a gene changes auxin sensitivity and enhances salt resistance in tobacco. Acta Physiol. Plant. 32, 997–1003.

Foster, R.J., Mcrae, D.H. and Bonner, J. (1952) Auxin-induced growth inhibition: a natural consequence of two-point attachment. Proc. Natl Acad. Sci. USA, 38, 1014–1022.

Gao, P., Bai, X., Yang, L., Lv, D., Pan, X., Li, Y., Cai, H. et al. (2011) osa-MIR393: a salinity- and alkaline stress-related microRNA gene. Mol. Biol. Rep. 38, 237–242.
Park, S.Y., Shijavi, R., Krans, J.V. and Luthe, D.S. (1996) Heat-shock response in heat-tolerant and non tolerant variants of *Agrostis palustris* Huds. *Plant Physiol.* **111**, 515–524.

Quetsch, C., Hong, S.W., Vierling, E. and Lindquist, S. (2000) Heat shock protein 101 plays a crucial role in thermotolerance in Arabidopsis. *Plant Cell*, **12**, 479–492.

Raghothama, K.G., Sheikh, A.H. and Sinha, A.K. (2014) Regulation of MAP kinase signaling cascade by microRNAs in *Oryza sativa*. *Plant Signal. Behav.* **9**, 125–135.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B. and Bartel, D.P. (2002) Prediction of plant microRNA targets. *Cell*, **110**, 513–520.

Sato, Y. and Yokoya, S. (2008) Enhanced tolerance to drought stress in transgenic rice plants overexpressing a small heat-shock protein, *sHSP17.7*. *Plant Cell Rep.* **27**, 329–334.

Shao, H.B., Chu, L.Y., Jaleel, C.A., Manivannan, P., Panneerselvam, R. and Shao, M.A. (2009) Understanding water deficit stress-induced changes in the basic metabolism of higher plants – biotechnologically and sustainably improving agriculture and the ecorenvironment in arid regions of the globe. *Crit. Rev. Biotechnol.* **29**, 131–151.

Shibasaki, K., Uemura, M., Tsurumi, S. and Rahman, A. (2009) Auxin response in Arabidopsis under cold stress: underlying molecular mechanisms. *Plant Cell*, **21**, 3823–3838.

Shimizuka, K. and Yamaguchi-Shinozaki, K. (2007) Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.* **58**, 221–227.

Shimizuka, K., Yamaguchi-Shinozaki, K. and Seki, M. (2003) Regulatory network of gene expression in the drought and cold stress responses. *Curr. Opin. Plant Biol.* **6**, 410–417.

Si-Ammour, A., Windels, D., Arn-Boudoirdes, E., Kutter, C., Alhas, J., Meins, F. Jr. and Vazquez, F. (2011) miR393 and secondary siRNA regulate expression of the TR1/AFB2 auxin receptor clade and auxin-related development of Arabidopsis leaves. *Plant Physiol.* **157**, 683–691.

Sinha, A.K., Jaggi, M., Raghothama, K. and Tuteja, N. (2011) Mitogen-activated protein kinase signaling in plants under abiotic stress. *Plant Signal. Behav.* **6**, 196–203.

Stamm, P. and Kumar, P.P. (2013) Auxin and gibberellin-dependent Arabidopsis SMALL AUXIN UP RNA3 regulates hypocotyl elongation in the light. *Plant Cell Rep.* **32**, 759–769.

Sun, W., Van, M.M. and Verbruggen, N. (2002) Small heat shock proteins and molecular chaperones in the abiotic stress response. *Front. Plant Sci.* **6**, 233–251.
are presented as means of three biological replicates, and error bars represent SD.

**Figure S2** Generation and molecular analysis of transgenic lines expressing Osa-miR393a. (a) Schematic diagram of the Osa-miR393a overexpression construct, p35S-Osa-miR393a/p35S-Hyg. Osa-miR393a is under the control of the Cauliflower mosaic virus (CaMV) 35S promoter and linked to the hygromycin resistance gene, Hyg, driven by the CaMV 35S promoter. LB; Left border; RB, right border; NOS, nopaline synthase terminator. (b) PCR analysis of the Hyg gene using genomic DNA of wild-type and transgenic plants to detect transgene insertion into the host genome. (c) Semi-quantitative RT-PCR analysis of the primary Osa-miR393a transcripts in transgenics. (d) Stem-loop RT-qPCR analysis to detect the expression of mature miR393 in transgenic and wild-type plants. Data are presented as means of three biological replicates, and error bars represent SD. Asterisks indicate a significant difference of expression levels between wild-type and each transgenic line at *P < 0.001 by Student’s t-test.

**Figure S3** The phenotype of two-month-old wild-type and transgenic plants initiated from the same number of tillers and grown in 6-inch pots.

**Figure S4** Overexpression of Osa-miR393a leads to enhanced salt tolerance in transgenic turfgrass plants. (a) Wild-type controls and three transgenic lines initiated from the same number of tillers were fully developed in pots for 10 weeks under normal conditions in growth room before salt stress application. (b) Performance of wild-type and transgenic plants subjected to 250 mM NaCl treatment for 10 days. (c) Performance of wild-type and transgenic plants 7 days after recovery from a 10 days salt treatment.

**Figure S5** Overexpression of Osa-miR393a leads to enhanced heat tolerance in transgenic turfgrass plants. Wild-type controls and three transgenic lines were fully developed in pots for 10 weeks under normal conditions in growth room. Plants were then transferred to the growth chamber and subjected to heat stress at 40 °C in the light and 35 °C in the dark for 15 days. The relative humidity in the chamber was 60%–80%. (a) and (c) Wild-type controls and three transgenic lines initiated from the same number of tillers were trimmed to the same height before the heat stress test. (b) Performance of wild-type and transgenic plants grown at 25 °C in the light and 17 °C in the dark for 15 days. (d) Performance of wild-type and transgenic plants 10 days after recovery from a 15 days heat treatment at 40 °C in the light and 35 °C in the dark.

**Figure S6** Shoot and root biomass change in wild-type (WT) and transgenic (TG) plants subjected to heat stress. (a) Performance of wild-type plants and three independent transgenic lines 13 days after growth under 25 °C/17 °C or 40 °C/35 °C. (b) Shoot fresh weight and (c) dry weight in wild-type and three independent transgenic lines 13 days after growth under 25 °C/17 °C or 40 °C/35 °C. (d) Root fresh weight and (e) dry weight in wild-type and three independent transgenic lines 13 days after growth under 25 °C/17 °C and 40 °C/35 °C. Data are presented as means of three biological replicates, and error bars represent SD.

**Figure S7** Amino acid sequences alignment of AsTIR1, AtTIR1 and OsTIR1.

**Figure S8** Amino acid sequences alignment of AsAFB2-1, AsAFB2-2, AtAFB2 and OsAFB2.

**Figure S9** Differential gene expression in transgenic (TG) vs. wild-type (WT) control plants. Volcano plot shows log2 FC of TG vs. WT data sets at normal conditions.

**Figure S10** GO enrichment analysis in transgenic (TG) vs. wild-type (WT) control plants.

**Figure S11** Expression levels of (a) AsNHX1 and (b) AsVP1 in wild-type (WT) and transgenic (TG) plants examined via qRT-PCR. Their differential expression between wild-type (WT) and transgenic (TG) plants was initially revealed by RNA-seq data. Data are presented as means of three biological replicates, and error bars represent SD. Asterisks indicate a significant difference of expression levels between the wild-type and transgenic plants at *P < 0.001 by Student’s t-test.

**Figure S12** Relative expression levels of the two putative miR393 targets of creeping bentgrass were determined in leaves under IAA treatment. Data are presented as means of three biological replicates, and error bars represent SD. Asterisks indicate significant differences of gene expression levels between untreated and stress-treated leaf tissues: *P < 0.05; and ***P < 0.001 by Student’s t-test.

**Table S1** Primer sequences used in this study.

**Table S2** The sequence of putative target genes for miR393 in creeping bentgrass, target sites are shown in red.