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Original Research Paper

MmZFP1 Response to Abiotic Stress in the Invasive Plant *Mikania micrantha*

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ABSTRACT

Mikania micrantha is one of the most problematic invasive alien species in China, and has had a serious economic and environmental impact. In the majority of plant genera, Cys2/His2-type zinc-finger proteins (C2H2-type ZFPs) are involved in abiotic stress responses. In the present study, *MmZFP1*, a two-fingered C2H2-type ZFP gene, was cloned and characterized from *Mikania micrantha*. MmZFP1 has two C2H2-type finger domains and other three conserved regions, including a B-box, an L-box and a DLN-box, which may be located in the nucleus. In our experiments *MmZFP1* was strongly expressed in roots, but weakly in stems and leaves. In *Mikania micrantha* the expression of *MmZFP1* could be induced by ABA, by dehydration and by high salinity. Observations of the phenotypes indicated that constitutive expression of *MmZFP1* in transgenic lines resulted in growth retardation and this could be correlated with the expression level of *MmZFP1*. Over-expression of *MmZFP1* in *Arabidopsis thaliana* can up-regulate the expression levels of the drought stress tolerance genes including *ABF3* and *ABF4* under normal growing conditions, which results in improved tolerance to drought. Both of the stress tolerance genes are involved in ABA-dependent pathways. Thus, our results suggest that MmZFP1 plays a key role in drought stress resistance, and this information is helpful in providing further understanding the molecular mechanism of the high degree of environmental adaptability of *Mikania micrantha*.

INTRODUCTION

Invasive alien plants can change the structure and function of ecosystems due to their high degree of adaptability, morphological plasticity, competitive ability and potential to modify soil properties (Shen et al. 2015). *Mikania micrantha*, which is native to Central and South America, is one of the 10 most problematic weeds and one of the 100 worst invasive alien species in the world (Yan et al. 2011, Lowe et al. 2000). *Mikania micrantha* has spread to southern China after 1910 and does not have any natural enemies in these regions. Since the 1980s, it has started to spread and invade widely and has become one of the most serious invasive alien species in China causing substantial damage to natural ecosystems and biodiversity (Zhang et al. 2004). As *Mikania micrantha* grows very fast, it has been called the "mile-a-minute" weed and is endangering plantation crops and forested ecosystems, causing serious economic and environmental impacts (Shen et al. 2015, Chen et al. 2013). *Mikania micrantha* has brought a large number of economic losses in Guangdong province of China (Yan et al. 2011).

Growth and development of plants may be affected by a variety of environmental stresses such as drought, salt and cold, plants respond and adapt to these stresses with a number of physiological and developmental changes to increase their stress tolerance (Abe et al. 1997, Sun et al. 2014). In these processes, numerous transcription factors (TFs) play key roles through regulating the expression of the stress-inducible genes. These families of TFs involved in the response to abiotic stress include bZIP, AP2/ERF, NAC, WRKY, MYB, Cys2/His2-type zinc-finger proteins (C2H2-type ZFPs), and bHLH (Lindemose et al. 2013).

C2H2-type ZFPs are also called TFIIIA-type ZFPs or classical ZFPs. The conserved sequence motif of $CX_{2-4}CX_3FX_3QALGGHX_{3-5}H$ is a particular structural feature. Plant C2H2-type ZFPs have one to four zinc-fingers. Furthermore, the long spaces with a diversity of lengths between the adjacent fingers are significant features distinguishing plants from other eukaryotes (Takatsuji 1999, Ciftci-Yilmaz & Mittler 2008). Numerous reports have implicated C2H2-type ZFPs in abiotic stress tolerance. Four C2H2-type ZFP genes in *Arabidopsis*: *AZF1*, *AZF2*, *AZF3* and *STZ* (*ZAT10*) are involved in the water stress response in an ABA-dependent or independent pathway to regulate the expression of the downstream genes (Sakamoto et al. 2000). SCOF-1, a TFIIIA-type ZFP found in soybean, is induced by cold stress and is

able to enhance cold tolerance in transgenic plants (Kim et al. 2001). *CAZFP1* is constitutively expressed in *Arabidopsis thaliana* to improve its tolerance of drought (Kim et al. 2004). Constitutive expression of *Zat7* confers tolerance to salinity stress in *Arabidopsis*, and the EAR-domain of C2H2-type ZFPs plays a key role in this defence response (Ciftci-Yilmaz et al. 2007). Overexpression of *ZFP252*, which is a TFIIIA-type ZFP gene from rice, enhances drought and salt tolerance (Xu et al. 2008). Overexpression of a C2H2-type ZFP gene *ZmZF1* from maize improves the tolerance to salt and drought in transgenic *Arabidopsis* (Huai et al. 2009). *AhZFP1*, which is the first C2H2-type ZFP gene reported for the peanut, is able to respond to salt stress in the peanut's roots, stems and leaves (Pan et al. 2010). In tobacco, expression of *StZFP1,* driven by the *Arabidopsis* stress-inducible *rd29A* promoter, increases the tolerance of transgenic plant to salt stress (Tian et al. 2010). ZFP182, a C2H2-type ZFP from rice, is involved in ABA-induced antioxidant defence (Zhang et al. 2012). AtZAT6 plays an essential role in plant responses to abiotic stress by activating the expression of *CBF* and *PR* genes, and this modulates drought, salt and cold resistance (Shi et al. 2014).

In this study, we cloned and characterized a C2H2-type ZFP gene, *MmZFP1* (KF516995) from *Mikania micrantha*. The expression of this gene in *Mikania micrantha* could be induced by ABA, dehydration and high salinity. In addition, the overexpression of *MmZFP1* conferred tolerance to drought in *Arabidopsis thaliana*. Our results indicate that MmZFP1 could play an important role in drought tolerance.

MATERIALS AND METHODS

Plant materials and growth conditions: *Mikania micrantha* seeds were collected in Guangzhou, Guangdong Province, in the south of China. The *Mikania micrantha* seeds were sown into pots containing a mixture of nutrient soil and vermiculite (1:1) and cultured in a growth chamber at 25°C ± 2°C under 16 h light (8000 lux) and 8 h dark.

Seeds of *Arabidopsis thaliana* (ecotype Columbia) were surface-sterilized with 75% ethanol and 2.5% NaClO solution, and then sowed in potting soil (nutrient soil: vermiculite = 1:2) or on MS culture medium containing 2% (w/v) sucrose. After 4°C vernalization in darkness for 3 days, the seeds were cultured in a growth chamber at 22°C ± 2°C with 70% relative humidity under 16 h light (8000 lux) and 8 h dark.

Cloning of MmZFP1: Total RNA of *Mikania micrantha* was isolated using RNApure Plant Kit (Cwbio, Beijing, China). In order to obtain the full-length cDNA encoding MmZFP1, primers of RACE PCR were designed, which were based on *Mikania micrantha* TSA (HQ316791) and homologous sequences of *Nicotiana tabacum* (AF053077), *Chrysanthemum x morifolium* (JQ040514) and *Vitis vinifera* (XM_002264266). 3' RACE PCR was performed with two nested gene-specific sense primers, 3'GSP1 and 3'GSP2, following the specification of the 3'-Full RACE Core Set with PrimeScript™ RTase (Takara, Shiga, Japan). 5'RACE cDNA was obtained by using the 5' RACE System for Rapid Amplification of cDNA Ends, according to the manufacturer's instructions (Invitrogen, CA, USA). Specific first-strand cDNA synthesis was performed with the antisense primer 5'GSP1, and then the tailed cDNA was used as a template for amplification with the adapter primer and two nested primers 5'GSP2 and 5'GSP3. All of the amplified products were purified and cloned in pMD 18-T vector (Takara, Shiga, Japan) for sequencing. All details of these primers are provided in Table 1.

Sequence analysis: The full-length cDNA and amino acid sequences were analysed using DNAMAN and DNAClub software. Sequence features, including the molecular mass and pI, were evaluated using the ExPASy-Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Amino acid sequence alignment was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Vector construction and plant transformation: Total RNA of *Mikania micrantha* was isolated using RNApure Plant Kit (Cwbio, Beijing, China), and was then reverse transcribed into first strand cDNA by using TIANScript RT Kit (Tiangen, Beijing, China). The complete open reading frame of *MmZFP1* was amplified by RT-PCR with primer pairs 1300-ZFP1-F/1300-ZFP1-R (Table 1) to generate *Pst* I and *Sal* I restriction sites at the 5' end and 3' end, respectively. Then the resulting DNA fragment was cloned into a Super 1300 vector containing a hygromycin-resistant selectable marker. This insert fragment was expressed under the control of a super promoter (Gong et al. 2002).

The *Arabidopsis* transformation was carried out using the floral dip technique (Zhang et al. 2012). The constructed

Table 1: Primer sequences used for cloning of *MmZFP1* and vector construction.

Primer Name	Sequence (5' -3')
3' GSP1	TCGACCTTGAAACCTAGTGG
3' GSP2	TCCATCTGCCACCGGTCGTT
5' GSP1	CCGGTGGCAGATGGAGCACT
5' GSP2	TCCACTAGGTTTCAAGGTCG
5' GSP3	GACGTAGACGGGTGGTCATC
1300-ZFP1-F[a]	CACACTGCAGATGGCACTTGAAGCTCTG
1300-ZFP1-R[b]	ACCAGTCGACTGTAACTCATAACGTCGCC

Note: [a] *Pst* I restriction site is underlined. [b] *Sal* I restriction site is underlined.

Table 2: Primer sequences used for real-time PCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
MmZFP1[a]	AGCCAGCCACCGGAAAAATG	GGAGTTGTTGCCGTCGTAGT
Mmactin7	AGGCGGGATTTGCTGGT	TACCTCTTTTGGACTGGGCTTC
MmZFP1[b]	GCGGTGACAGCTACAGTTTC	TTCCACTAGGTTTCAAGGTCGA
ACT2	AAGTCTTGTTCCAGCCCTCG	TTTGCTCATACGGTCAGCGA
ABF3	ACACAGTGCCAGGAAGTGAA	GATCAGGTGACATCTGGCTT
ABF4	AATCAGCCTCATCCACAGCA	CGGTTCCTCCGTAACTAGCTAAT
DREB2A	AAAGGTAAAGGAGGACCAGA	GCCAAAGGACCATACATAGC
DREB2B	TGTATGAAGGGTAAAGGAGGAC	TGAGGGAAGTTAAGACGAGC
CBF1	CAACTTCGCTGACTCGGCTTG	ATCGTCTCCTCCATGTCCAGG
CBF2	CGGTGATTACAGTCCGAAGC	CACACCCACTTACCGGAGTT
CBF3	CGGTAAGTGGGTTTGTGAGG	GCAAGTTGATTCCGGGATTCG

Note: [a]Primers used for real-time PCR where the template was isolated from *Mikania micrantha*. [b]Primers used for real-time PCR where the template was isolated from transgenic plants.

vector harbouring *MmZFP1* was introduced into *Arabidopsis* using *Agrobacterium tumefaciens* (GV3101) transformation. The seeds of the transgenic plants were selected by hygromycin (25 mg/L) and those seedlings which had survived were further confirmed by PCR and real-time PCR (Kim et al. 2004, Wang et al. 2011, Shi et al. 2014). In this case, the PCR test based on the genomic DNA of these plants, was used to confirm the positive transformants. Real-time PCR was used to detect the expression level of *MmZFP1* in the transgenic lines. *ACT2* (At3g18780) was used as the internal control.

Abiotic stress treatment and quantitative real-time PCR: 8-week-old seedlings of *Mikania micrantha* were subjected to the following abiotic stress experiments. The seedlings were removed to solutions containing 100 μM abscisic acid (ABA), 20% (w/v) polyethylene glycol (PEG6000), or 300 mM NaCl, respectively. All samples were taken at the designated time intervals after treatment, immediately frozen in liquid nitrogen, and stored at -80°C for later total RNA isolation.

The total RNA of *Mikania micrantha* was isolated using RNApure Plant Kit (Cwbio, Beijing, China), and total RNA of *Arabidopsis thaliana* was isolated using TRNzol Reagent (Tiangen, Beijing, China). Then, the total RNA was reverse transcribed into first strand cDNA by using FastQuant RT Kit with gDNase (Tiangen, Beijing, China). Real-time PCR was performed by using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara, Shiga, Japan) with an ABI 7500 thermocycler (Applied Biosystems, CA, USA).

For real-time PCR to detect the expression pattern of *MmZFP* in *Mikania micrantha*, *Mmactin*7 (EY456955) was used as an internal control to normalize the *MmZFP1* expression level. Three biological and three technical replicates were performed. In order to monitor the transcript levels of the drought-responsive genes, the seeds of Line 5 and Line 12 were selected using hygromycin (25 mg/L), then 6-week-old plants with similar levels of growth in each line were picked out. The expression levels of *MmZFP1* and of a number of drought-responsive genes in these selected transgenic plants were detected by real-time PCR. These drought-responsive genes included *ABF3*, *ABF4* (*AREB2*), *DREB2A*, *DREB2B*, *CBF1* (*DREB1B*), *CBF2* (*DREB1C*), *CBF3* (*DREB1A*). *ACT2* was used as an internal control. The experiment was performed with three biological repeats and three technical replicates.

All the specific primers for real-time PCR were specifically designed and are listed in Table 2, while the primers for *Mmactin7* and *DREB2A* were based on previous studies (Li et al. 2010, Shi et al. 2014). Because the specificity of the *MmZFP1* primers used for *Mikania micrantha* was not appropriate in *Arabidopsis*, a further set of primers was designed for *Arabidopsis*. Expression levels for all the candidate genes were determined by using the $2^{-\Delta\Delta CT}$ method (Zhang et al. 2011).

Plant drought stress resistance assay: Water deficit stress treatment was followed as previously described (Shi et al. 2013), but with a number of alterations. Essentially, 3-week-old plants were subjected to a water deficit condition by withholding water for three weeks, and then re-watered for one week. Plants were grown in common conditions to provide controls. The survival rate was scored after re-watering treatment.

RESULTS AND DISCUSSION

Cloning and sequence analysis of *MmZFP1*: We cloned the full-length cDNA sequence of *MmZFP1* and this coding sequence has been deposited in GenBank under accession no. KF516995. The entire cDNA sequence of *MmZFP1* consists of 1,010 bp with a single open reading frame (ORF) of 816 bp, encoding a protein of 271 amino acid residues and

a calculated molecular mass of 29.0 kDa with pI 9.18.

Multiple sequence alignment analysis of two-fingered C2H2-type ZFPs from various species has revealed the structural features of MmZFP1 (Fig. 1). There are five conserved regions in this protein. MmZFP1 contained two C2H2-type zinc-finger domains that have the $CX_{2-4}CX_3FX_3QALGGHX_{3-5}H$ sequence motif. QALGGH is a highly conserved motif in these C2H2-type zinc-finger domains, and this is a prominent structural feature of TFIIIA-type ZFPs from plants (Takatsuji 1999). The B-box with a consensus sequence KXKRSKRXR represents a potential nuclear localization signal (NLS) (Lawrence et al. 2014). Furthermore, there is also a hydrophobic leucine-rich region with a core sequence EXEXXAXCLXXL (L-box), which is probably related to protein-protein interaction or to maintaining the folded structure (Sakamoto et al. 2000). The DLN-box located in the C-terminal region is an EAR motif that is involved in transcription repression (Ohta et al. 2001). The EAR motif-containing C2H2-type ZFPs can act as repressors in plants (Ciftci-Yilmaz & Mittler 2008), and play a key role in regulation of the defence response to abiotic stresses (Ciftci-Yilmaz et al. 2007).

Transcript levels of *MmZFP1*: A tissue-specific expression pattern of *MmZFP1* was detected in the roots, stems, and leaves of *Mikania micrantha* (Fig. 2). The results showed that *MmZFP1* was strongly expressed in roots, but weakly in stems and leaves.

To investigate the expression of *MmZFP1* in response to abiotic stress, *Mikania micrantha* seedlings were treated with different chemical inductions. After ABA treatment, the transcription of *MmZFP1* was up-regulated rapidly to a maximum at 1 h, and then showed a downward trend until 6 h. At 12 h, the expression of *MmZFP1* reached a second high (Fig. 3a). Under treatment with PEG6000 to induce drought stress, *MmZFP1* transcript levels continued to increase from 0 h-12 h, achieved their peak at 12 h (Fig. 3b). In the salt stress treatment with NaCl (300 mM), the expression level of *MmZFP1* increased from 0 h to 6 h, and remained at a similar level until 12 h, but then peaked at 18 h (Fig. 3c). These results indicated that *MmZFP1* is regulated by ABA, and induced after drought and salt stress treatments. Therefore, MmZFP1 is likely to play a role in plant responses to abiotic stress.

Effect on plant growth and development: After screening for hygromycin tolerance, the putative transgenic plants were further confirmed by PCR of their genomic DNA. Thus, we obtained ten *MmZFP1* transgenic T1 lines. Subsequently, the relative transcript level of *MmZFP1* of eight of the transgenic T1 lines was verified by real-time PCR (Fig. 4a). The result showed that the *MmZFP1* expression level of

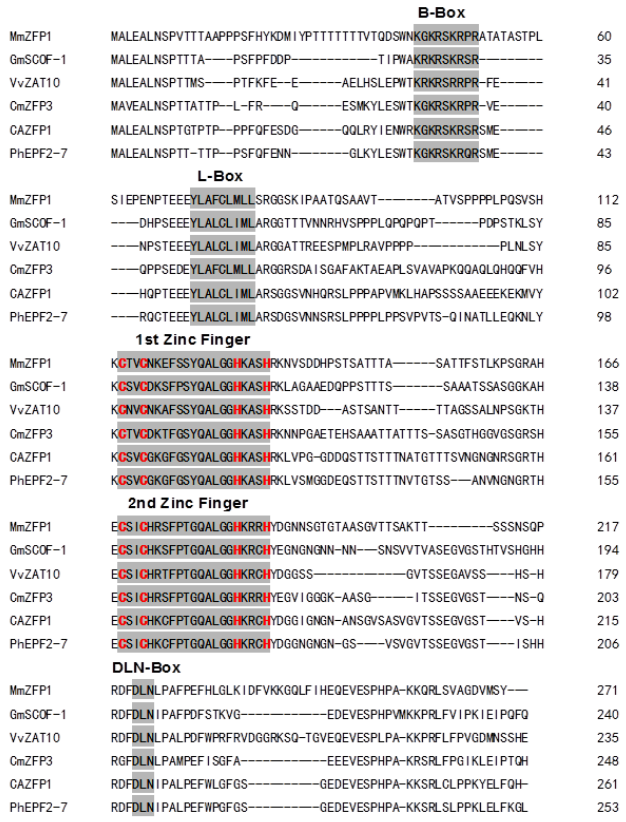

Fig. 1: Multiple sequence alignment analysis of MmZFP1 and its homologous sequences from other species. *Mikania micrantha* MmZFP1 (AHA80146), *Capsicum annuum* CAZFP1 (AAQ10954), *Glycine max* GmSCOF-1 (AAB39638), *Vitis vinifera* VvZAT10 (XP_002264302), *Chrysanthemum x morifolium* CmZFP3 (AFF57513), *Petunia x hybrid* PhEPF2-7 (BAA05079). The conserved regions of the two zinc-finger motifs, B-box, L-box and DLN-box are shaded grey. The Cys (C) and His (H) residues in the zinc-finger motif are highlighted in red. The gaps introduced to maximize alignment are indicated by dashes.

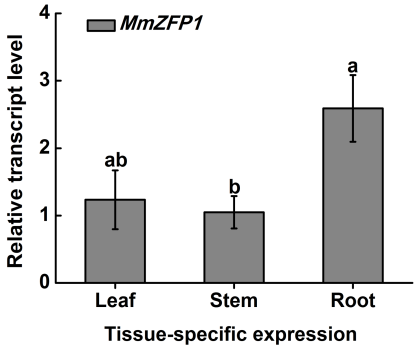

Fig. 2: Tissue-specific expression pattern of *MmZFP1* in *Mikania micrantha*. Data are the mean values of three biological repeats. Error bars show standard errors (SE). The different letters located above each column indicate the significant differences at $P < 0.05$ according to Duncan's multiple range test.

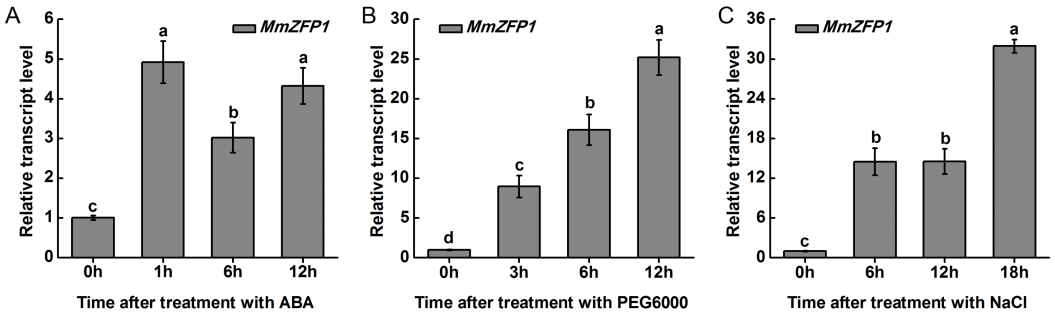

Fig. 3: The expression pattern of *MmZFP1* after stress treatments. (A) The expression level of *MmZFP1* after ABA treatment. (B) The expression level of *MmZFP1* after PEG6000 treatment. (C) The expression level of *MmZFP1* after NaCl treatment. Data are mean values of three biological repeats. Error bars show standard errors (SE). The different letters located above each set of columns indicate the significant differences at $P < 0.05$ according to Duncan's multiple range test.

Line 12 was the highest. The next highest was Line 5. The expression levels of the other lines were much lower. Line 12 and Line 5 were selected to perform the subsequent experiments.

The growth of the *MmZFP1* transgenic lines was distinctly retarded, compared with that of wild type plants. 3-week-old *MmZFP1* transgenic plants of Line 5 and Line 12 were typified by short root length, which was different from that of the wild type (Fig. 4b). In addition, constitutive expression of *MmZFP1* reduced the size of the whole plants and also led to smaller leaves (Fig. 4c). As shown in Fig. 4d, there were significant differences between the 10-week-old wild-type and the *MmZFP1*-transgenic lines. Plants of Line 5 and Line 12 exhibited reduced plant height, greater leaf numbers in the rosette, and fewer siliques and fioral organs when compared with wild type plants. These observations indicate that constitutive expression of *MmZFP1* had repressed the growth and development of the transgenic lines.

Constitutively expressed two-fingered C2H2-type ZFP genes could result in the suppression of growth and development that has been described in some previous studies. Both in terms of root length and whole plant size, *CAZFP1* transgenic T2 plants are markedly smaller than those of the wild type (Kim et al. 2004). Ciftci-Yilmaz et al. (2007) found that a high level of *ZAT7* expression resulted in growth suppression. In the study of Vogel et al. (2005), the level of *ZAT12* expression was considered the key factor that negatively affected the growth and development of transgenic plants. The overexpression of *AtZAT6* shows pleiotropic phenotypes with curly leaves, small-sized plants and reduced size of the fioral organs and siliques. The differences in pleiotropic phenotypes between the transgenic lines and the wild type are caused by different expression levels of *ZAT6* (Shi et al. 2014). In our study, the *MmZFP1* expression level of Line 12 was higher than that of Line 5, which resulted in the much more severe suppression of growth and development in Line 12 compared with Line 5. These results demonstrate that the suppression of growth and development may be correlated with the expression level of *MmZFP1* in the transgenic lines.

Overexpression of *MmZFP1* in Arabidopsis enhances drought stress tolerance: Because the expression of *MmZFP1* was transcriptionally induced by ABA and abiotic stresses, including high salinity and dehydration (Fig. 3a-c), we investigated the capacity of *MmZFP1*-overexpression lines to respond to drought stress. In this study, 3-week-old wild type, and *MmZFP1*-overexpression, plants were withheld water for three weeks, and then recovered for one week. As shown in Fig. 5(a-d), after drought stress treatments, *MmZFP1*-overexpressing plants showed better growth than the wild type. Wild type plants became severely wilted and impaired by the drought stress treatment. On the contrary, *MmZFP1*-overexpression lines were relatively healthier after suffering this severe drought stress. After re-watering, plants of Line 5 and Line12 had therefore exhibited higher survival rates than the wild type. These results suggested that *MmZFP1*-overexpression conferred improved resistance to drought stresses in *Arabidopsis* and that MmZFP1 functioned as a positive regulator involved in the acclimation.

MmZFP1 positively regulates the expression of drought stress tolerance genes: It is a hallmark of plant acclimation that numerous stress-responsive genes are induced under abiotic stress. To provide further insight into the molecular mechanism of *MmZFP1* in such drought responses, we monitored the transcript levels of drought-responsive genes by real-time PCR analysis. These genes include *ABF3* (At4g34000), *ABF4* (*AREB2*, At3g19290), *DREB2A* (At5g05410), *DREB2B* (At3g11020), *CBF1* (*DREB1B*, At4g25490), *CBF2* (*DREB1C*, At4g25470) and *CBF3*

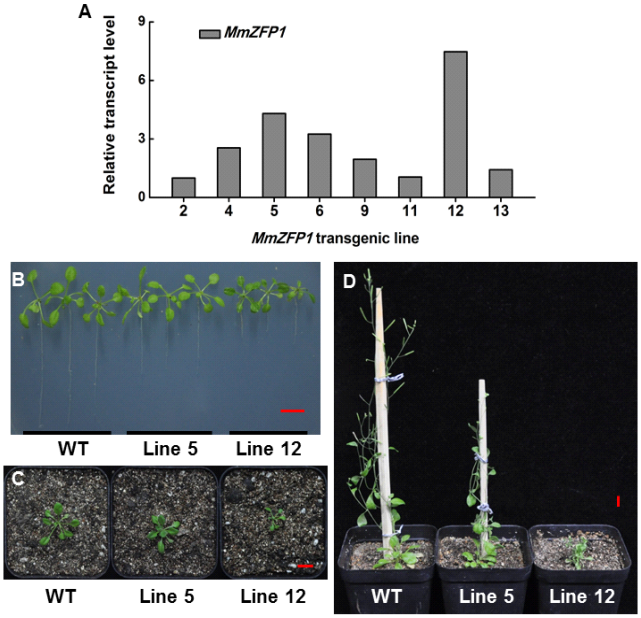

Fig. 4: Developmental phenotypes of *MmZFP1*-overexpressing plants. (A) Relative transcript level of *MmZFP1* in transgenic T1 lines. Data are mean values of three technical replications. (B) Root growth of 3-week-old wild type and *MmZFP1*-overexpressing plants. (C) Morphology of 4-week-old wild type and *MmZFP1*-overexpressing plants in soil. (D) 10-week-old wild-type and *MmZFP1*-overexpressing plants. Red bars represent 1 cm.

(*DREB1A*, At4g25480). As shown in Fig. 6, *ABF3* and *ABF4* were significantly up-regulated in the transgenic lines, compared with the wild type.

A water-deficit condition induces a range of physiological and biochemical responses in plants. In these processes, a number of stress-inducible genes play roles in the initial stress response or in establishing the plants' stress tolerance (Shinozaki & Yamaguchi-Shinozaki 2007). There are ABA-independent and ABA-dependent regulatory systems governing drought-inducible gene expression (Shinozaki et al. 2003). ABFs function in the ABA signalling pathway. Overexpression of *ABF3* and *ABF4* in *Arabidopsis* results in reduced transpiration and enhanced drought tolerance (Kang et al. 2002). DREBs (CBFs) are on the ABA-independent pathway (Yamaguchi-Shinozaki & Shinozaki 2005). DREB2 proteins including DREB2A and DREB2B, function as trans-acting factors under dehydrating conditions. Expression of *DREB2A* and *DREB2B* is induced by dehydration and high-salt stress (Liu et al. 1998). Overexpression of *CBF3* activates the expression of stress tolerance genes and results in an improved tolerance to drought (Kasuga et al. 1999). *CBF1* and *CBF2* are homologues of *CBF3*. In *Arabidopsis*, *CBF1*, *CBF2* and *CBF3* play key roles in the response to abiotic stresses including

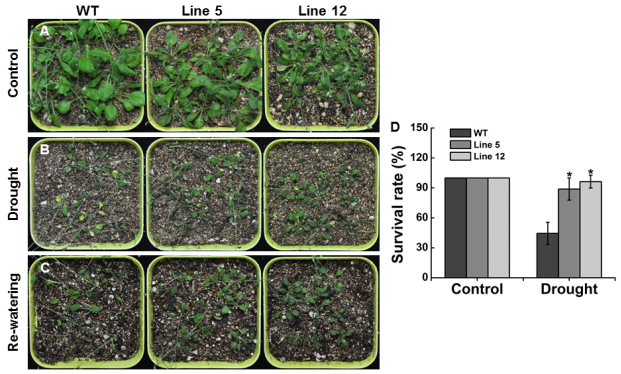

Fig. 5: Modulation of *MmZFP1* expression affects drought stress resistance. (A) Plants under normal conditions served as a control. (B) 3-week-old plants without watering for three weeks. (C) Plants after drought stress treatment were re-watered for one week. (D) Survival rate of the wild type and *MmZFP1*-overexpressing plants after recovery for one week from drought stress treatments. Data are mean values of three replications. Error bars show standard deviation (SD). Asterisks located above a set of columns indicate a significant difference of $P < 0.05$ compared with the wild type.

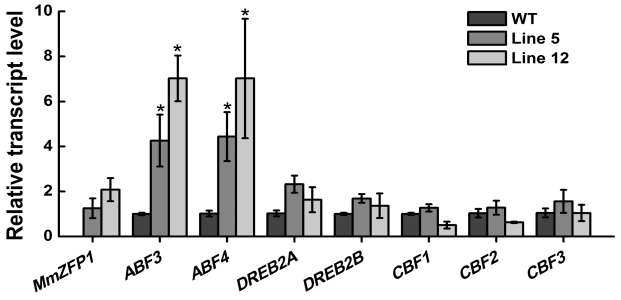

Fig. 6: Expression levels of drought stress responsive genes in 6-week-old plants. Data are mean values of three biological repeats. Error bars show standard errors (SE). Asterisks located above a set of columns indicate a signiûcant difference of $P < 0.05$ compared with the wild type.

cold, drought and high salt (Novillo et al. 2012). In the present study, *ABF3* and *ABF4* were conspicuously up-regulated in transgenic *Arabidopsis* under normal growing conditions. ABF3 and ABF4 are classed as basic leucine zipper (bZIP) transcription factors (Kang et al. 2002). ABRE is an important *cis*-acting element in the ABA-dependent signalling pathway. ABFs can bind to ABRE to activate ABA-dependent gene expression (Shinozaki & Yamaguchi-Shinozaki 2007). These expression profiles implied that MmZFP1 might function in ABA-dependent regulatory systems to enhance drought stress tolerance in the transgenic plants.

CONCLUSIONS

In this study, *MmZFP1* responded to ABA, drought and salt stress in *Mikania micrantha*. Overexpression of *MmZFP1*

activated the expression of stress tolerance genes including *ABF3* and *ABF4* under normal growing conditions, and resulted in improved tolerance to drought. These two stress tolerance genes are involved in ABA-dependent signalling pathways. The above results imply that MmZFP1 functions as a key regulator in ABA-dependent signalling pathways, and this information should prove valuable in our further understanding of the molecular mechanism behind the high degree of adaptability of *Mikania micrantha*.

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REFERENCES

Abe, H., Yamaguchi-Shinozaki, K. and Urao, T. 1997. Role of *Arabidopsis* MYC and MYB homologs in drought-and abscisic acid-regulated gene expression. Plant Cell, 9(10): 1859-1868.

Chen, B.M., Peng, S.L. and D'Antonio, C.M. 2013. Non-additive effects on decomposition from mixing litter of the invasive *Mikania micrantha* HBK with native plants. PloS One, 8(6): e66289.

Ciftci-Yilmaz, S. and Mittler, R. 2008. The zinc finger network of plants. Cellular and Molecular Life Sciences, 65(7-8): 1150-1160.

Ciftci-Yilmaz, S., Morsy, M.R. and Song, L. 2007. The EAR-motif of the Cys2/His2-type zinc finger protein Zat7 plays a key role in the defense response of Arabidopsis to salinity stress. Journal of Biological Chemistry, 282(12): 9260-9268.

Gong, Z., Lee, H. and Xiong, L. 2002. RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. Proceedings of the National Academy of Sciences, 99(17): 11507-11512.

Huai, J., Zheng, J. and Wang, G. 2009. Overexpression of a new Cys2/His2 zinc finger protein ZmZF1 from maize confers salt and drought tolerance in transgenic *Arabidopsis*. Plant Cell, Tissue and Organ Culture (PCTOC), 99(2): 117-124.

Kang, J. Y., Choi, H. I. and Im, M. Y. 2002. *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell, 14(2): 343-357.

Kasuga, M., Liu, Q., Miura, S. and Yamaguchi-Shinozaki, K. 1999. Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotechnology, 17(3): 287-291.

Kim, J. C., Lee, S. H. and Cheong, Y. H. 2001. A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. The Plant Journal, 25(3): 247-259.

Kim, S. H., Hong, J.K. and Lee, S.C. 2004. *CAZFP1*, Cys2/His2-type zinc-finger transcription factor gene functions as a pathogen-induced early-defense gene in *Capsicum annuum*. Plant Molecular Biology, 55(6): 883-904.

Lawrence, S.D., Novak, N.G. and Jones, R.W. 2014. Herbivory responsive C2H2 zinc finger transcription factor protein StZFP2 from potato. Plant Physiology and Biochemistry, 80: 226-233.

Li, D. M., Staehelin, C. and Wang, W. T. 2010. Molecular cloning and characterization of a chitinase-homologous gene from *Mikania micrantha* infected by *Cuscuta campestris*. Plant Molecular Biology Reporter, 28(1): 90-101.

Lindemose, S., O'Shea, C. and Jensen, M. K. 2013. Structure, function and networks of transcription factors involved in abiotic stress responses. International Journal of Molecular Sciences, 14(3): 5842-5878.

Liu, Q., Kasuga, M. and Sakuma, Y. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought-and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell, 10(8): 1391-1406.

Lowe, S., Browne, M. and Boudjelas, S. 2000. 100 of the world's worst invasive alien species: a selection from the global invasive species database. Auckland, New Zealand: Invasive Species Specialist Group.

Novillo, F. Medina, J. and Rodríguez-Franco, M. 2012. Genetic analysis reveals a complex regulatory network modulating CBF gene expression and *Arabidopsis* response to abiotic stress. Journal of Experimental Botany, 63(1): 293-304.

Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H. and Ohme-Takagi, M. 2001. Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell, 13(8): 1959-1968.

Pan, L., Yang, Q. and Chi, X. 2010. AhZEP1, a cDNA encoding C2H2-type zinc finger protein, induced by salt stress in peanut (*Arachis hypogaea* L.). In: Bioinformatics and Biomedical Engineering (iCBBE), 2010, 4th International Conference, IEEE, pp. 1-7.

Sakamoto, H., Araki, T. and Meshi, T. 2000. Expression of a subset of the *Arabidopsis* Cys 2/His 2-type zinc-finger protein gene family under water stress. Gene, 248(1): 23-32.

Shen, S., Xu, G. and Clements, D. R. 2015. Suppression of the invasive plant mile-a-minute (*Mikania micrantha*) by local crop sweet potato (*Ipomoea batatas*) by means of higher growth rate and competition for soil nutrients. BMC Ecology, 15(1): 1.

Shi, H., Wang, X. and Ye, T. 2014. The Cysteine2/Histidine2-type transcription factor zinc finger of *Arabidopsis thaliana 6* modulates biotic and abiotic stress responses by activating salicylic acid-related genes and *C*-repeat-binding factor genes in *Arabidopsis*. Plant Physiology, 165(3): 1367-1379.

Shi, H., Ye, T. and Chen, F. 2013. Manipulation of arginase expression modulates abiotic stress tolerance in *Arabidopsis*: Effect on arginine metabolism and ROS accumulation. Journal of Experimental Botany, 64(5): 1367-1379.

Shinozaki, K. and Yamaguchi-Shinozaki, K. 2007. Gene networks involved in drought stress response and tolerance. Journal of Experimental Botany, 58(2): 221-227.

Shinozaki, K., Yamaguchi-Shinozaki, K. and Seki, M. 2003. Regulatory network of gene expression in the drought and cold stress responses. Current Opinion in Plant Biology, 6(5): 410-417.

Sun, Y., Luo, W. and Sun, S. 2014. Exogenous hydrogen sulphide protection of *Cucurbita ficifolia* seedlings against $NaHCO_3$ stress. Nature Environment and Pollution Technology, 13(2): 381-384.

Takatsuji, H. 1999. Zinc-finger proteins: the classical zinc finger emerges in contemporary plant science. Plant Molecular Biology, 39(6): 1073-1078.

Tian, Z. D., Zhang, Y. and Liu, J. 2010. Novel potato C2H2-type zinc finger protein gene, StZFP1, which responds to biotic and abiotic stress, plays a role in salt tolerance. Plant Biology, 12(5): 689-697.

Vogel, J. T., Zarka, D. G. and Van Buskirk, H. A. 2005. Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. The Plant Journal, 41(2): 195-211.

Wang, Q., Li, F. and Zhang, X. 2011. Purification and characterization of a CkTLP protein from *Cynanchum komarovii* seeds that confers antifungal activity. PloS One, 6(2): e16930.

Xu, D. Q., Huang, J. and Guo, S. Q. 2008. Overexpression of a TFIIIA-type zinc finger protein gene ZFP252 enhances drought and salt tolerance in rice (*Oryza sativa* L.). FEBS Letters, 582(7): 1037-1043.

Yamaguchi-Shinozaki, K. and Shinozaki, K. 2005. Organization of cis-acting regulatory elements in osmotic-and cold-stress-responsive promoters. Trends in Plant Science, 10(2): 88-94.

Yan, Y., Huang, Y. and Fang, X. 2011. Development and characterization of EST-SSR markers in the invasive weed *Mikania micrantha* (Asteraceae). American Journal of Botany, 98(1): e1-e3.

Zhang, B., Yang, Y. and Chen, T. 2012. Island cotton Gbve1 gene encoding a receptor-like protein confers resistance to both defoliating and non-defoliating isolates of *Verticillium dahliae*. PloS One, 7(12): e51091.

Zhang, H., Ni, L. and Liu, Y. 2012. The C2H2-type zinc finger protein ZFP182 is involved in abscisic acid-induced antioxidant defense in rice. Journal of Integrative Plant Biology, 54(7): 500-510.

Zhang, L. Y., Ye, W. H. and Cao, H. L. et al. 2004. *Mikania micrantha* HBK in China - An overview. Weed Research, 44(1): 42-49.

Zhang, Y., Wang, Q. and Zhang, X. 2011. Cloning and characterization of an annexin gene from *Cynanchum komarovii* that enhances tolerance to drought and *Fusarium oxysporum* in transgenic cotton. Journal of Plant Biology, 54(5): 303-313.