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Repression of ARF10 by microRNA160 plays an important role in the mediation of leaf water loss

Xin Liu[1,2,3] · Xiufen Dong[1,2,3] · Zihan Liu[1,2,3] · Zihang Shi[1,2,3] · Yun Jiang[1,2,3] ·
Mingfang Qi[1,2,3] · Tao Xu[1,2,3] · Tianlai Li[1,2,3]

Abstract Solanum lycopersicum *auxin response factor 10* (*SlARF10*) is post-transcriptionally regulated by *Sl-miR160*. Overexpression of a *Sl-miR160*-resistant *SlARF10* (*mSlARF10*) resulted in narrower leaflet blades with larger stomata but lower densities. *35S:mSlARF10-6* plants with narrower excised leaves had greater water loss, which was in contrast to the wild type (WT). Further analysis revealed that the actual water loss was not consistent with the calculated stomatal water loss in 35S:*mSlARF10-6* and the WT under the dehydration treatment, indicating that there is a difference in hydraulic conductance. Pretreatment with abscisic acid (ABA) and $HgCl_2$ confirmed higher hydraulic conductance in *35S:mSlARF10,* which is related to the larger stomatal size and higher activity of aquaporins (AQPs). Under ABA treatment, *35S:mSlARF10-6* showed greater sensitivity, and the stomata closed rapidly. Screening by RNA sequencing revealed that five AQP-related genes, fourteen ABA biosynthesis/signal genes and three stomatal development genes were significantly altered in *35S:mSlARF10-6* plants, and this result was verified by qRT-PCR. The promoter analysis showed that upregulated AQPs contain AuxRE and ABRE, implying that these elements may be responsible for the high expression levels of AQPs in *35S:mSlARF10-6*. The three most upregulated AQPs (*SlTIP1-1-like, SlPIP2;4* and *SlNIP-type-like*) were chosen to confirm AuxRE and ABRE function. Promoters transient expression demonstrated that the *SlPIP2;4* and *SlNIP-type-like* AuxREs and *SlPIP2;4* and *SlTIP1-1-like* ABREs could significantly enhance the expression of the GUS reporter in *35S:mSlARF10-6*, confirming that AuxRE and ABRE may be the main factors inducing the expression of AQPs. Additionally, two upregulated transcription factors in *35S:mSlARF10-6, SlARF10* and *SlABI5-like* were shown to directly bind to those elements in an electromobility shift assay and a yeast one-hybrid assay. Furthermore, transient expression of down-regulated *ARF10* or up-regulated *ABI5* in tomato leaves demonstrated that *ARF10* is the direct factor for inducing the water loss in *35S:mSlARF10-6*. Here, we show that although *SlARF10* increased the ABA synthesis/signal response by regulating stomatal aperture to mitigate water loss, *SlARF10* also influenced stomatal development and AQP expression to affect water transport, and both act cooperatively to control the loss of leaf water in tomato. Therefore, this study uncovers a previously unrecognized leaf water loss regulatory factor and a network for coordinating auxin and ABA signalling in this important process. In an evolutionary context, *miR160* regulates *ARF10* to maintain the water balance in the leaf, thus ensuring normal plant development and environmental adaptation.

Xin Liu and Xiufen Dong have contributed equally to this work.

✉ Tao Xu
  syauxutao@qq.com

✉ Tianlai Li
  tianlaili@126.com

1 Horticulture Department, College of Horticulture, Shenyang Agricultural University, No. 120 Dongling Road, Shenhe District, Shenyang 110866, Liaoning, People's Republic of China

2 Key Laboratory of Protected Horticulture of Ministry of Education, Shenyang, Liaoning Province, People's Republic of China

3 Key Laboratory of Protected Horticulture of Liaoning Province, Shenyang, Liaoning Province, People's Republic of China

Introduction

Both the growth and geographic distribution of plants are
greatly affected by the environment, but plants also undergo
favourable genetic and phenotypic alterations in response to
abiotic and biotic changes in the environment. Of the abiotic
stresses, the supply of water plays a key role in determining
plant growth, and a range of physiological and biochemi-
cal changes occur in plants to enable them to survive water
stress (Shinozaki and Yamaguchi-Shinozaki 2007). These
responses include stomatal closure, repression of cell growth
and photosynthesis, and activation of respiration. Through
long-term natural selection, water loss must be minimized
for plant growth to adapt to drought stress, such as through
thick cuticles, concave stomata, rich leaf hairs and narrow
blade surface areas (Mulroy and Rundel 1977).

The leaf is the key organ involved in the evaporation of
water from plants, and two factors affect the loss of water
through the leaves. The stoma is one of the major factors
influencing leaf water loss, and stomatal closure regulates
water loss by transpiration. The stomatal conductance to
water vapour in the mature leaf is predominantly deter-
mined by stomatal size and density (Franks et al. 2009), and
stomatal size is important because it uniquely defines the
upper limit of density (Franks and Beerling 2009). A simi-
lar measure of stomatal size, when multiplied by density,
was found to be correlated with leaf hydraulic conductance
(Sack et al. 2003). The cleavage of *growth-regulating factor*
(*GRF*) target genes by *miR396*, which is involved in *Ara-
bidopsis* leaf development, can affect stomatal density and
thus increase endurance under drought (Liu et al. 2009).

On its way through the leaf from the xylem to the sto-
mata, water can either move through cell walls or pass from
cell to cell across different tissues. Although both pathways
are likely used to some degree, there is increasing evidence
that living cells contribute substantially to the overall leaf
hydraulic conductance (K_{leaf}). Transcellular water flow is
facilitated and regulated by water channels in the mem-
branes, named aquaporins (AQPs) (Heinen et al. 2009), and
they facilitate the rapid, passive exchange of water across
cell membranes and are responsible for up to 95 % of the
water permeability of plasma membranes (Henzler et al.
2004). The AQPs are particularly important for maintaining
the large amounts of water being transported because they
respond differently to water stress (Hachez et al. 2006b;
Heinen et al. 2009). Several studies have shown that water
stress can alter leaf AQP expression and activity, which
modifies plant adaptations to water stress (Aharon et al.
2003; Lian et al. 2006).

Abscisic acid (ABA) is a plant stress hormone and one
of the most important signalling molecules in the regulation
of leaf water loss (Peleg and Blumwald 2011). Water stress
increases the ABA plant hormone by strongly inducing the
expression of a 9-cis-epoxycarotenoid dioxygenase (NCED)
gene, which is a key enzyme in ABA biosynthesis (Iuchi et
al. 2001). Moreover, carotenoids are precursor molecules for
ABA, and the formation of GGPP is a key step in the carot-
enoids biosynthetic pathway. This step is catalysed by gera-
nylgeranyl pyrophosphate synthase (GGPS). Induction of
carotenoid biosynthesis contributes to ABA production upon
salt stress in *Arabidopsis* (Ruiz-Sola et al. 2014; Li et al.
2008). ABA curtails transpirational water loss by promoting
stomatal closure and inhibiting stomatal opening (Acharya
and Assmann 2009); moreover, the application of the hormone
could decrease the relative loss of water content in plants
through decreased leaf transpiration (Raphael and Maarten
2001). ABA controls leaf water deficits by both changing the
stomatal apertures (for example, the stomata of ABA-treated
plants exhibit more complete closure than control plants) and
by modifying the hydraulic conductivity in plants (Franks and
Farquhar 2001; Hose et al. 2000; Parent et al. 2009; Thomp-
son et al. 2007). Under water stress, stomatal closure may be
mediated by ABA or the hydraulic conductivity of the xylem
vessels (Brodribb and Holbrook 2003; Cochard et al. 2002).
Drought-induced ABA diminishes the osmotic water perme-
ability of bundle-sheath cells by downregulating the activity
of their AQPs (Shatil-Cohen et al. 2011), and the ABA signal
is also reportedly involved in water stress. *ABA INSENSI-
TIVE 1* (*ABI1*) and *ABI2* are also crucial for ABA-mediated
stomatal regulation (Gosti et al. 1999; Merlot et al. 2001), and
the overexpression of *ABRE-BINDING FACTOR* (*ABF3*) or
AREB2/*ABF4* caused ABA hypersensitivity, reduced the tran-
spiration rate and enhanced drought tolerance in transgenics
(Kang et al. 2002). Over-expression of *ABI5* could enhance
plant resistance to drought stress. ABRE is known to mediate
gene regulation by ABI/ABF, and AuxRE is known to medi-
ate gene regulation by ARFs (Fricke et al. 2013). High ABA
content, or a strong ABA signal, is thought to be positively
correlated with plant resistance to water stress.

Potential cross-talk between auxin and ABA has been
suggested by a number of studies in a variety of plants.
Auxin response factor 2 (*ARF2*) was induced by ABA, and
the *arf2* mutants displayed enhanced ABA sensitivity during
seed germination and primary root growth, suggesting that
ARF2 represses the ABA signalling pathway (Wang et al.
2011). Auxin controls seed dormancy through the stimula-
tion of abscisic acid signalling by *ARF10* and *ARF16*, which
induces ARF-mediated *ABI3* activation in *Arabidopsis* (Liu
et al. 2013). The differential expression of drought-related
proteins, which is ABA-dependent, and the related drought-
adaptive responses in auxin mutant *AXR2* and/or *AXR1*
indicate cross-talk between auxin- and ABA-signalling in

Arabidopsis (Bianchi et al. 2002). Auxin may participate in the positive regulation of drought stress resistance through the regulation of root architecture and the expression of ABA-responsive genes (Shi et al. 2014).

Recently, *miR160* has been reported to play a major role in the ABA response in plants by mediating the accumulation of *ARF10, 16* and *17* at the mRNA level. The negative regulation of *ARF10* by *miR160* affects ABA sensitivity and may play a role in auxin-ABA cross-talk (Liu et al. 2007). However, the role of *ARF10/miR160* in ABA dependence and its direct role in the adaptation to water stress are still unclear.

The *Sl-miR160a*-resistant (*mSlARF10*) transgenic lines offer a good system to study the relationship between water loss in tomato leaves and the regulation of *ARF10* auxin–ABA crosstalk by *miRNA160*. In this study, we report that *35S:mSlARF10-6* transgenic plants developed narrow leaflet blades, a theoretically highly efficient water-saving leaf type, but exhibited rapid water loss under the water stress treatment. Further, this study revealed that the density and size of individual stoma are altered in *35S:mSlARF10-6*, and its water loss capacity may be determined by the stomatal factor. However, with prolonged treatment time, water loss is significantly not in accordance with the stomatal factor because *mSlARF10* accumulates greater ABA content and sensitivity under water stress, which leads to more sensitive stomata closure. The use of a special AQP inhibitor in $HgCl_2$-pretreated leaves supported our hypothesis that AQP may play an important role in the overexpression of *mSlARF10* in leaf water loss. Real-time-PCR and RNA sequencing revealed that *mSlARF10* overexpression could enhance expression of ABA synthesis gene, the ABA signal factor and AQP expression, and *cis*-element analysis indicated upregulated AQPs promoters contain ABRE and AuxRE. Transient expression analysis indicated that AuxRE majorly contributes to enhanced AQP expression, while ABRE can simultaneously enhance or depress its expression. The yeast-one hybrid assay and EMSA determined that the ARF10 could directly bind to the relevant site of the promoter sequence during the upregulation of AQP. The upregulated ABI5 in *35S:mSlARF10* could also directly bind to the upregulated AQP, which contains ABRE. To verify whether *SlARF10* enhances water loss by boosting *ABI5* expression, transient expression of down-regulated *ARF10* or up-regulated *ABI5* in tomato leaves was carried out. The results demonstrated that '*ARF1*' could descrease the water loss rate and the expression of most AQPs, while *ABI5+* could significantly decrease the water loss rate at the ealier stage of water stress, although the expression of some AQPs was indeed enhanced. Here, we show that although *SlARF10* increased the ABA synthesis/signal response by regulating stomatal aperture to mitigate water loss, *SlARF10* also directly influenced stomatal development and AQP expression to affect water transport, and both actions cooperated to control the loss of water in tomato leaves. In an evolutionary context, *miR160* regulates *ARF10* to maintain the water balance in the leaf, thus ensuring normal plant development and enviormental adaptation.

Materials and methods

Plant materials and growth conditions

Tomato cv. MP1 plants (Barg et al. 1997) were grown under standard greenhouse conditions at 24 °C/18 °C (day/night) with a photoperiod of 16 h light/8 h dark. The *35S:mSlARF10-6* transgenic plants were supplied by Arazi (Hendelman et al. 2012). All of the study plants were grown under the same conditions; whole plants were grown and watered daily for 6 weeks.The samples were all obtained from the primary leaflets of the fifth leaf in each plant.

Measurement of water loss

To measure water loss, each of the 35 fully expanded, true leaves of the 6–8-week-old MP1 and *35S:mSlARF10-6* plants were detached and weighed at different times (0, 0.5, 1, 2, 6, 12 and 24 h) to determine the rate of water loss. Five leaves from each type of plant were selected at each of the time points, which were determined by consulting the literature (Kahn et al. 1993). For time course studies, leaves were weighed, and then placed on the bench to undergo dehydration for periods of 0–24 h, measured from the start of wilting. Leaf water loss ratio as a percentage of (fresh weight-dry weight)/fresh weight was calculated at the time of leaf sampling. At each time point, some leaves were frozen in liquid nitrogen and stored at −80 °C.

Leaf hydraulic conductance measurements

Leaf hydraulic conductance was measured using the evaporative flux method for three MP1 and three *35S:mSlARF10-6* plants. A filtered (0.2 μM) 20 mM KCl + 1 mM $CaCl_2$ solution (subsequently referred to as 'artificial xylem sap', AXS) was used for these measurements. Flow rate through leaves was measured with a balance (model CP 224S, Sartorius, Gottingen, Germany), which logged data to a computer every 30 s. The air was adequately stirred by a fan as explained by Sack and Scoffoni (2012). Leaves were illuminated with ~1000 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) at the leaf surface by an LED worklight. Leaf temperature was monitored by a thermocouple. Leaf water potential (ψ_{leaf}) was measured using a C-52 sample chamber in a Dewpoint Water Potential System (Psypro, Wescor, Utah, USA); leaf water potential was measured after 25 min of equilibration at 25–30 °C. Four replicates were performed for each plant in this experiment. For

hydrated leaves, the K_{leaf} was calculated as described previously (Sack and Scoffoni 2012) using the finalψ_{leaf} (ψ_{final}), which was determined at the end of the measurement of E (transpiration rate) which refers to the plant unit leaf area of transpiration of water within a certain amount of time. K_{leaf} is calculated as $E/-\Delta\psi_{leaf}$ (where $\Delta\psi_{leaf} = \psi_{final} -0$ MPa) and normalized by leaf area. Leaf area per square metre per hour is commonly used in transpiration water grams.

Application of ABA and HgCl$_2$

Non-stressed leaves were perfused for 2 h with artificial xylem sap (AXS, i.e., filtered 20 mM KCl + 1 mM CaCl$_2$ solution), AXS + 0.2 mM HgCl$_2$ or AXS + 50 μM ABA (Laur and Hacke 2014). Solutions were introduced into the transpiring leaf by immersing the petiole in 50 mL containers. The excised leaves were then bench-dried at the different time points, and the leaves were frozen in liquid nitrogen and stored at −80 °C.

RNA extraction, reverse transcription-PCR and real-time quantitative PCR assay

Total RNA from the same plant material samples as previously described for the physiological experiments samples was extracted using TRIzol (Takara, Dalian, China) followed by an RNase-free treatment (Takara). The RNA (4 μg) was pretreated with RQ1 DNase I (Promega, Madison, WI, USA) to remove contaminating genomic DNA. Total RNA concentrations were measured using a NanoDrop unit (Thermo Fisher Scientific, Waltham, MA, USA), and RNA quality was checked using nondenaturing agarose gel electrophoresis. Total DNA-free RNA (2 μg) was used for complementary DNA (cDNA) synthesis in a total volume of 40 μL, and the resulting cDNA was used as the template for RT-PCR. The PCR for the mRNAs was carried out in 20 μL reactions containing 2 μL of cDNA synthesis reaction mixture, 400 nM of each primer, and 10.5 μL of SYBR Green PCR Master Mix (TianGen Biotech, Beijing, China) on an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The temperature program began with 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 45 s at 57 °C; the melting temperature of the amplified product was determined to verify the presence of a specific product.

The main feature of mature miRNA expression detection is the RT primer, which has a stem-loop structure and a consensus sequence that effectively binds to the miRNA 3′ end. The PCR for the mature miRNAs was carried out in 25 μL reactions containing 2.5 μL of cDNA synthesis reaction mixture, 400 nM of each primer, and 12.5 μL of SYBR Green PCR Master Mix. The PCR temperature program began with 10 s at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. In addition, each measurement was repeated using three technical replicates, in which the RNA samples were mixed with three biological replicates.

To obtain accurate target mRNA expression profiles, we designed primers that flanked the cleavage sites using Primer Premier 5.0 (Premier, Canada). Sense primers were designed upstream of the miRNA cleavage sites and antisense primers were designed downstream of the cleavage sites to ensure that we would be able to detect the remaining intact mRNA levels after miRNA cleavage. For the normal genes, the sequences were obtained from the NCBI. The primer design involved selection of a specific sequence from the whole gene sequence. In addition, each measurement was repeated using three biological replicates. The primers for each gene are listed in Supplementary Table 1.

Deep sequencing

Tomato leaflet samples were collected from MP1 and *35S:mSlARF10-6* at the same stage and position on the plants, and an Illumina MiSeq library was constructed according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Magnetic beads with poly T oligos attached were used to purify the mRNA from the total RNA. Fragmentation buffer was added to cleave the mRNA into short fragments, and the fragments were used to synthesize first-strand cDNA using random hexamerprimers. The cDNA was transformed into double-stranded cDNA with RHase H and DNA polymerase I, and a paired-end library was constructed from the synthesized cDNA with a Genomic Sample Prep Kit (Illumina). Fragments of desirable lengths were purified with a QIAquick PCR Extraction Kit (Qiagen), end repaired and linked with sequencing adapters (Margulies et al. 2005). AMPureXP beads were used to remove the unsuitable fragments, and the sequencing library was the constructed by PCR amplification. After being checked with PicoGreen staining and fluorospectrophotometry and then quantified with an Agilent 2100, the multiplexed DNA libraries were mixed in equal volumes to a normalized 10 nM concentration. The sequencing library was then sequenced with the Illumina Miseq platform (Shanghai Personal Biotechnology Cp., Ltd., Shanghai, China).

Statistical analysis of the stoma characteristics

The epidermis was separated from the 20 true leaves from the MP1 and *35S:mSlARF10-6* plants to analyse stomatal density and size, respectively. The stomatal number and size from a field of view of a given area were measured under light microscopy (Zeiss Observer A1). The stomatal density for each leaf was calculated as the mean of four fields of view at 20× magnification, and the stomatal morphological parameters were measured as the mean of the 20 stomatal complexes for each leaf. Means were compared by Fisher's LSD test at a significance level of 0.05. The stomatal morphological parameters

were stomatal length, stomatal aperture width, stomata aperture area (length×width×3.14) and stomata pore depth. The maximum stomatal conductance to water vapour (g_{wmax}, in mol m^{-2} s^{-1}) was estimated as follows (Franks and Beerling 2009; Franks and Farquhar 2001):

$$g_w = \frac{daD}{V\left(1 + \frac{p}{2}\sqrt{\frac{a}{p}}\right)},$$

d = diffusivity of water in air (m^2 s^{-1}) (2.55×10^{-5}); D = stomata per unit epidermal area (m^{-2}); a = mean stomatal pore area (m^2); V = molar volume of air (m^3 mol^{-1}) (22.4×10^{-3}); and l = depth of the stomatal pore (approximated as W/2 for guard cells). The environmental conditions were consistent; thus, d and V are the same for both the wild-type (WT) and *35S:mSlARF10-6* plants.

Quantification of ABA

ABA was quantified using the following ELISA protocol (Yang et al. 2001). The samples were homogenized in liquid nitrogen and extracted in cold 80% (v/v) methanol with butylated hydroxytoluene (1 mmol L^{-1}) overnight at 4°C. The extracts were collected after centrifugation at 10,000×g (4°C) for 20 min and then passed through a C18 Sep-Pak cartridge (Waters, Milford, MA) and dried in N$_2$. The residues were dissolved in PBS (0.01 mol L^{-1}, pH 7.4) to determine the levels of ABA. Microtitration plates (Nunc) were coated with synthetic ABA-ovalbumin conjugates in NaHCO$_3$ buffer (50 mmol L^{-1}, pH 9.6) and left overnight at 37°C. Ovalbumin solution (10 mg mL^{-1}) was added to each well to block non-specific binding, and after incubation for 30 min at 37°C, standard ABA, samples and antibodies were added and incubated for an additional 45 min at 37°C. The antibodies against ABA were obtained as described by Yang et al. (2001). Then, horseradish peroxidase-labelled goat anti-rabbit immunoglobulin was added to each well and incubated for 1 h at 37°C. Finally, the buffered enzyme substrate (orthophenylenediamine) was added, and the enzyme reaction was carried out in the dark at 37°C for 15 min and then terminated using 3 mol L^{-1} H$_2$SO$_4$. The absorbance was recorded at 490 nm. Analyses of the enzyme-immunoassay data were performed as described by Yang et al. (2001). The experiment was performed with three biological replicates, and three technical replicates.

Promoter analysis

The upstream sequence for each significantly differentially expressed tomato gene was obtained using the method by Liu et al. (2014), and we used the 2.5 kb sequence that was the 5′ of the start codon. The sequence and start codon locations were obtained from http://solgenomics.net/, and ITAG v 2.4 and v 2.5 were used for the gene models and chromosome sequences, respectively. The sequences were analysed to predict the promoter element using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Electrophoretic mobility shift assays (EMSAs)

The ARF10 was expressed as a His fusion in Rosetta cells, and ARF10 was proteolytically cleaved from the His tag during the purification. The oligonucleotides used to introduce pET30 were induced by IPTG and purified by Ni-NTP Spin Columns (Qiagen, Germany). Double-stranded 50 oligonucleotides corresponding to the sequences from the −318 to −368 *Solyc06g011350.2* (GAGACA) and −1039 to −1089 *Solyc05g008080.1* (TGTCTC) promoters were used as probes. Respectively, the sequences of the probes were as follows:

1. F: 'AATAGTTGATGATCTTGCAAGCTCTGTCTTG CTCTAACAAGTACCTTGAC'; R: 'TTATCAACTAC TAGAACGTTCGAGACAGAACGAGATTGT TCATGGAACTG'
2. F:'TTTGTTGATTGTCTCGTTGATCTACAGAGGCT GGAAAAGCTAAGTATAAC'; R: 'AAACAACTAAC AGAGCAACTAGATGTCTCCGACCTTTTCGA TTCATATTG'.

The oligonucleotide probes were 5′-end-labeled with biotin, 2 μL of 100 μM double-stranded probes, 0.2 μL of 50 mM EDTA, 2 μL binding buffer, 1 μL of poly (dI-dC), and 1 μL 50% glycerol (700 ng/μL added to 2–3 μL to reach 1–2 μg). ARF10 protein was incubated for another 20 min at room temperature. The DNA–protein complexes were separated by electrophoresis through a 7% nondenaturing polyacrylamide gel in 0.5×TBE buffer for 40 min at 4°C, 100 V; transmembrane: 100 V and 38 mA for 30 min. The membrane was then exposed to UV crosslinking for 2 min.

Transient expression and quantitative fluorometric β-glucuronidase assays

In the assay, the three AQP genes with the most upregulated expression (*Solyc10g083880.1*, 'SlTIP1-1-like', *Soly06g011350.2*, 'SlPIP2;4' and *Solyc05g008080.1*, 'SlNIP-type like') were selected for analysis. The promoter sequence was PCR-amplified to obtain some special elements (PIP-a, PIP-b, PIP-d, NIP-a, NIP-b, NIP-d, TIP-a, and TIP-b), and the PIP-c and NIP-c gene fragments were synthesized by the Shaibaisheng Company (Beijing, China). For the transient expression of transcripts, partial *SlARF10* and full length *SlABI5* were cloned, and then transferred into pB7GWIWG2(II) and pBI121 respectively.

Agrobacteria harbouring the pBI101-AQPpromoters, pB7GWIWG2(II).0-ARF10 and pBI121-ABI5 were grown overnight in YEB medium and transferred to induction medium (Nimchuk et al. 2000) with 50 µM acetosyringone for 4 h until the OD_{600} reached 0.8–1.2. Bacteria were diluted with one volume of 10 mM MES at pH 5.6, 10 mM $MgCl_2$, and 150 µM acetosyringone and pressure infiltrated into the leaves of 3–4-week-old tomato plants. Agrobacterium-mediated expression was performed as described in Wroblewski et al. (2005) with the EH105 Agrobacterium strain carrying the GUS-intron transgene in pBI101. At least three plants and three leaves per plant were used for each experiment.

Quantitative measurements of GUS activity were performed according to Jefferson et al. (1987). For the quantitative GUS assay, WT and 35S:mSlARF10-6 leaves were dissected and collected in 1.5 mL microfuge tubes with approximately three leaves per sample, and the GUS substrate was 4-methylumbelliferyl-β-D-glucuronide. Protein concentrations of the leaf extracts were determined by the dye-binding method. The GUS activities were calculated in relative units of fluorescence produced per µg protein per hour.

Yeast one-hybrid (Y1H) assays

Y1H assays were performed using a Matchmaker Gold Yeast One-Hybrid System Kit (Clontech) according to the manufacturer's protocols. SlARF10 or SlABI5-like was ligated to pGADT7 to generate the AD-ARF/ABI construct. The fragments of the AQP gene promoter were ligated to the pAbAi vector to generate pAbAi-bait plasmids, which were then linearized and transformed into the Y1HGold yeast strain and selected with a plate containing selective synthetic dextrose medium lacking uracil. The AD-ARF/AD-ABI constructs were transformed into the Y1HGold strain containing pAbAi-bait and screened on an SD/-Ura/AbA plate. All transformations and screenings were performed three times.

In situ hybridizations

Templates for the generation of ribonucleic acid probes were constructed as follows. A fragment comprising nucleotides 255–554 of Solyc10g083880.1 was amplified using the primers shown in the Supplementary Information and ligated into pSPT18 (Roche). Plasmids were linearized with EcoRI or HindIII and transcribed with T7 RNA polymerase. RNA probes labelled with digoxigenin-UTP were prepared using a DIG RNA-labelling mix (Roche).

Plant material was collected from 6-week-old MP1 and 35S:mSlARF10-6, and the leaf tissue section preparation and hybridization of DIG-labelled probes were performed as described by Guerin et al. (2000). Probe quality was assessed using gel electrophoresis.

Results

Effects of miR160-resistant ARF10 overexpression on leaf morphology and water loss

The 35S:mSlARF10-6 plants displayed small leaflets with extremely narrow blades (Hendelman et al. 2012), and the leaf index (the ratio of the leaf length to the leaf width) was analysed to further assess leaf morphology. The results showed that the length of the 35S:mSlARF10-6 leaf blades was reduced only by an average of approximately 0.7%, which was not significant; however, the width was significantly reduced by more than 45% compared with the control (P < 0.01). As a result, the leaf index was increased from 2.05 ± 0.21 in the control plants to 6.38 ± 0.62 in the transgenic 35S:mSlARF10-6 plants (Table 1), and the length of the leaf petiole in 35S:mSlARF10-6 was also reduced relative to the control.

The shape of the leaf is a key factor for transpiration in this organ, and the leaf index is negatively related to leaf water loss (Liu et al. 2009). The WT leaves wilted with increased duration of the dehydration treatment (Fig. 1); the 35S:mSlARF10-6 also wilted with time but more seriously than the WT. The water loss percentage of the bench-dried WT leaves increased from 0 to 24 h under dehydration treatment and reached an approximate plateau (Fig. 2a). The water loss from 35S:mSlARF10-6 was also increased by dehydration treatment, but it was always higher than the WT. Therefore, overexpression of mSlARF10 may increase water loss from the leaves of transgenic plants

Table 1 Effect of the overexpression of miRNA160-resistant SlARF10 on leaf development

	Leaf length (mm)	Leaf width (mm)	Leaf index	Leaf petiole (mm)
WT	4.12 ± 0.34	2.01 ± 0.17	2.05 ± 0.21	1.1 ± 0.018
35S:mSlARF10-6	3.89 ± 0.29	0.61 ± 0.05**	6.377 ± 0.62**	0.9 ± 0.027**

Fully expanded fifth leaves of MP1 and 35S:mSlARF10-6 are characterized (n = 10). Data are means ± SD. Leaf index is the ratio of the length to the width of the leaf blade. The experiments were repeated three times
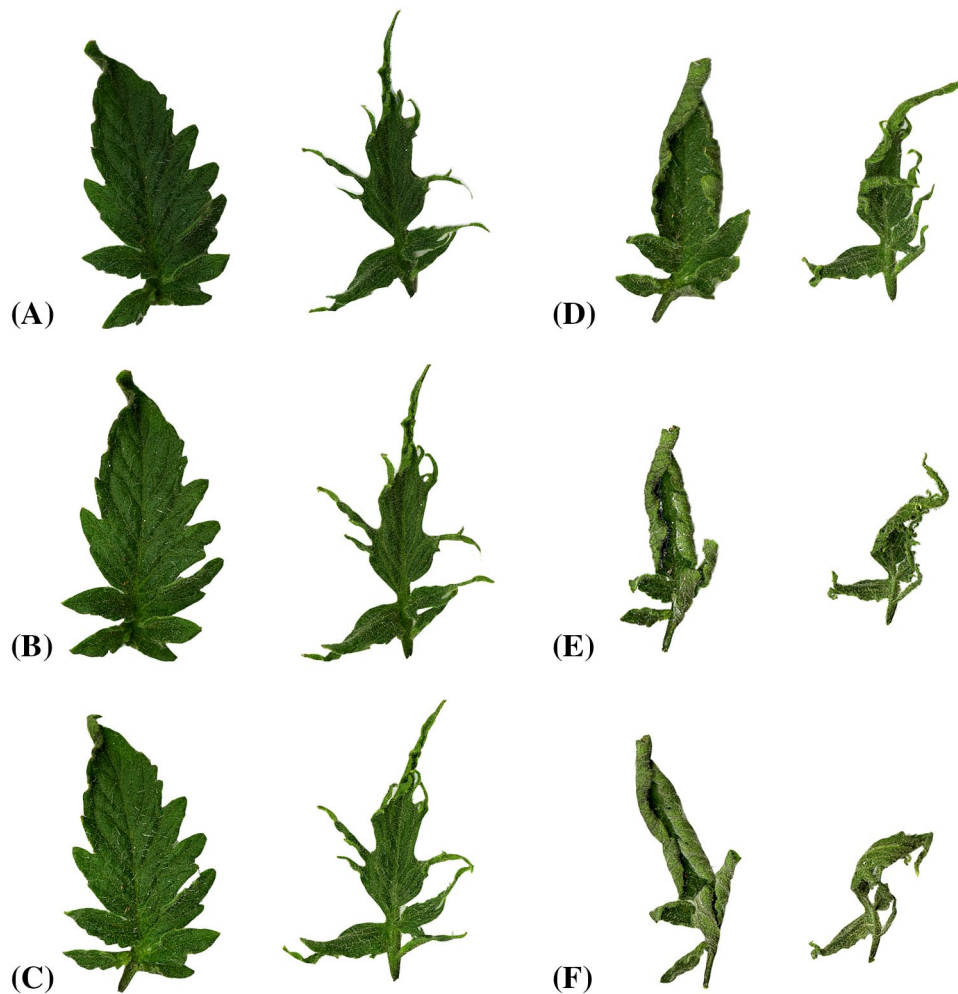
**Represents a significant difference, P < 0.01

Fig. 1 Comparison of leaf phenotypes between the WT and *35S:mSlARF10-6* plants. Detached leaves from 6-week-old plants were subjected to dehydration treatment. In each picture, the *left side* is the WT leaf, and the *right side* is the *35S:mSlARF10-6* leaf. **a** 0 h of dehydration treatment; **b** 1 h of dehydration treatment; **c** 2 h of dehydration treatment; **d** 6 h of dehydration treatment; **e** 12 h of dehydration treatment; **f** 24 h of dehydration treatment

by transpiration. The water fluxes in the leaves are regulated by the hydraulic conductivity of the leaf (K_{leaf}) in response to many abiotic stresses (Martre et al. 2002), so the excised leaves were immediately immersed in AXS. The transpiration rate (E) and water potential (ψ_{leaf}) of the leaves were measured, yielding a calculated K_{leaf} (ratio of E to ψ_{leaf}) for *35S:mSlARF10-6* of approximately 12.49 mM m^{-2} s^{-1} MPa^{-1} (Fig. 2b), which was significantly higher than that of the WT. These results showed that over-expression of *mSlARF10* not only changed the leaf shape but also influenced the hydraulic conductance.

Changes in stomatal morphology and conductance in *35S:mSlARF10-6*

Because the water loss and hydraulic conductivity were significantly different between *35S:mSlARF10-6* and WT,

the factors related to leaf water loss were analysed. Stomatal size, aperture and density were investigated in mature WT and *35S:mSlARF10-6* leaves. Furthermore, the over-expression of *mSlARF10* resulted in a significant decrease in stomatal density of approximately 30 ± 4 compared with 55 ± 4 in the WT (Fig. 3; Table 2). Stomatal density could affect water transpiration (Wang et al. 2007). The 'stomatal index' is defined as $100D/(D_E + D)$, where D is the density of the stoma and D_E is the number of epidermal cells per unit area (Royer 2001). The stomatal index in *35S:mSlARF10-6* was reduced to 2.94 compared with 3.07 in the WT, representing an approximate decrease of 4.3 % (Table 2). Based on the size of the stomata, the length of the WT stomata ranged from approximately 9.47 to 11.16, and the length in *35S:mSlARF10-6* ranged from 9.87 to 16.61 (Table 3). The size of the stomata was significantly larger in *35S:mSlARF10-6* than in the WT.
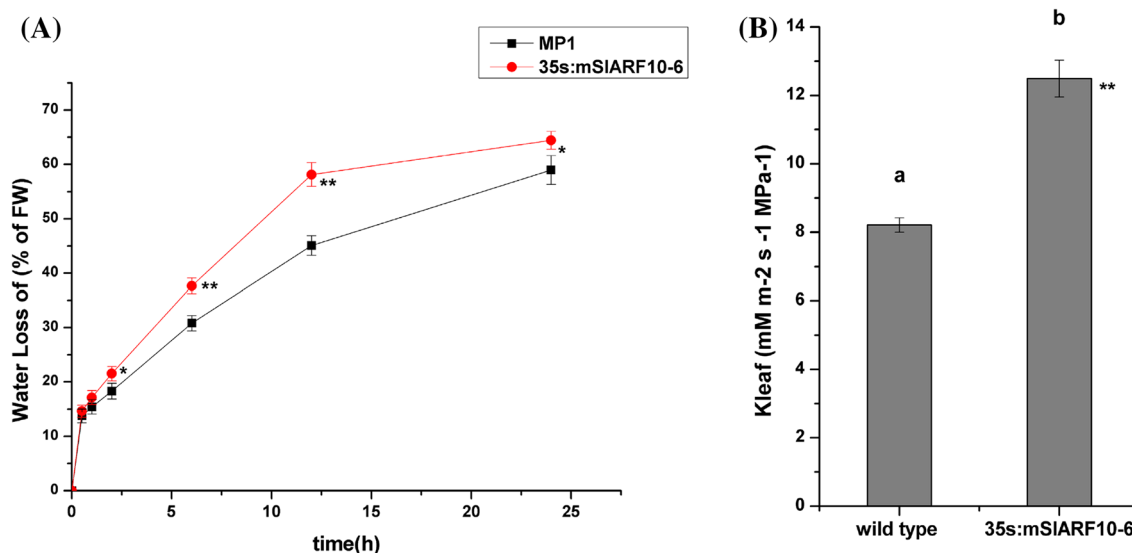
Fig. 2 Overexpression of *mSlARF10* increased the leaf water loss. **a** Water loss percentages from leaves of WT and *35S:mSlARF10-6* plants. Excised leaves from the fifth leaves of 6-week-old plants were assayed for water loss. Both WT and transgenic plants were grown under normal conditions. The excised leaves were bench-dried and weighed. **b** The leaf hydraulic conductance (K_{leaf}) of WT and *35S:mSlARF10-6* was measured after the leaves were xylem perfused with AXS after 2–3 h. Data represent means ± SD of four independent experiments. *Indicates a significant difference (P ≤ 0.05), and **indicates a highly significant difference (P ≤ 0.01)
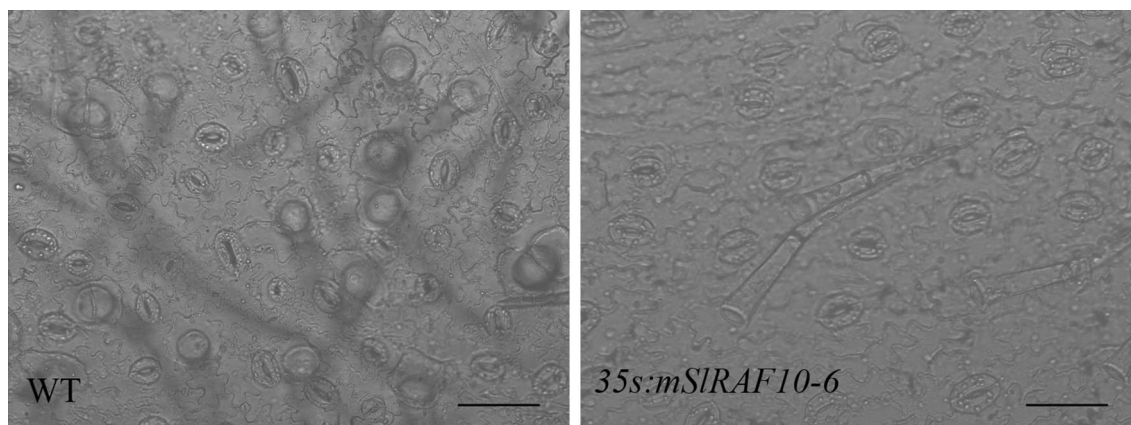

Fig. 3 Stomatal number decreased in transgenic *35S:mSlARF10-6* plants. Microscopic views of stomata in the fifth leaves of transgenic and WT plants. Six-week-old plants were used for all experiments. *Bar* 20 μm

Table 2 Stomatal and epidermal cell densities of the adaxial and abaxial surfaces, respectively, of 20 fully expanded true leaves of MP1 and *35S:mSlARF10-6* plants

	Stomatal density (D)	Epidermal cells density (D_E)	Stomatal index
WT	55 ± 4	124 ± 10	3.073 ± 0.063
35S:mSlARF10-6	30 ± 4**	72 ± 8**	2.941 ± 0.081

Data are means ± SD. Stomatal index is $100D/(D_E + D)$. Each leaf was observed in three visual fields under the microscope

**Represents a significant difference, P < 0.01

The aperture width in the WT decreased with dehydration treatment time, and the aperture in *35S:mSlARF10-6* decreased considerably with treatment time. The stomatal aperture area decreased from 101.51 to 60.02 in the WT with dehydration treatment (40.87 %), and the area was 54.92 % higher in *35S:mSlARF10-6* than in the WT (195.19 vs. 87.99). The range of decrease of the stomatal aperture area with dehydration treatment time was also greater in *35S:mSlARF10-6* than in the WT. Before 6 h of dehydration treatment, the stomatal aperture area decreased by 20.89 % in the WT, and the stomatal aperture area decreased by 45 % in *35S:mSlARF10-6*. Therefore,

Table 3 Stomatal sizes of adaxial and abaxial surfaces of leaves of MP1 and *35S:mSlARF10-6*

Variety	Treatment time (h)	Length (μm) (L)	Stomatal aperture width (μm) (W)	Stomatal pore area (μm²)	W/2 for guard cell (*l*) (μm)	g_w (mol m⁻² s⁻¹)
WT	0	11.162±0.394	2.804±0.101	101.518±6.798	11.014±0.121	0.3186±0.0012
	1	10.476±0.356	2.703±0.088	88.936±5.483	11.581±0.243	0.2792±0.0028
	2	10.410±0.459	2.636±0.089	87.757±5.516	11.992±0.109	0.2706±0.0009
	6	9.540±0.325	2.659±0.091	80.302±4.111	11.438±0.315	0.2593±0.0009
	12	10.868±0.421	2.256±0.074	77.579±4.394	10.945±0.396	0.2588±0.0013
	24	10.201±0.390	1.831±0.066	60.015±3.531	9.096±0.412	0.2355±0.0020
35S:mSlARF10-6	0	16.051±0.408**	4.129±0.251**	195.196±9.538**	7.573±0.189**	0.3340±0.0008**
	1	14.646±0.668**	3.430±0.215**	149.147±9.111**	7.164±0.256**	0.2831±0.0013
	2	16.618±0.339**	2.961±0.197	143.825±7.77**	7.263±0.333**	0.2745±0.0011**
	6	12.337±0.534**	3.008±0.239	107.245±7.60**	7.452±0.506**	0.2202±0.0008**
	12	9.870±0.502	3.270±0.312**	101.366±7.210**	8.732±0.419**	0.1960±0.0015**
	24	12.599±0.485**	2.595±0.250**	87.997±5.894**	8.911±0.328	0.1744±0.0023**

Twenty fully expanded 20 true leaves of MP1 and *35S:mSlARF10-6* plants respectively. Stomatal aperture area was calculated as'πLW'. Data are means±SD

**Represents a significant difference, P<0.01

Table 4 ABA contents of the leaves of MP1 and *35S:mSlARF10-6* were quantified at different dehydration treatment time points

Variety	Content of ABA in leaves(ng g⁻¹ Fw)					
Dehydration treatment time (h)	0	1	2	6	12	24
Control	58.9568±6.3452	62.3601±4.8139	68.4593±4.3734	72.5420±5.0347	73.2358±5.5556	74.6604±4.9293
35S:mSlARF10-6	91.2324±4.7561**	103.4384±4.7118**	110.6342±4.8616**	120.7728±4.0495**	90.8966±4.1186**	106.9422±5.5208**

Data are means±SD. The data are presented as three biological replicates

**Represents a significant difference, P<0.01

the *35S:mSlARF10-6* leaves were more sensitive to water stress than the WT leaves, as indicated by regulation of the stomatal aperture. Furthermore, the decrease in the area of the stomatal aperture before 6 h of dehydration treatment was greater in *35S:mSlARF10-6* than in the WT. The stomatal size, density and aperture were closely related to the stomata-dependent water loss, which was calculated according to previously established methods (Franks and Beerling 2009; Franks and Farquhar 2001). As calculated, the stomatal conductance to water vapour (g_w) gradually decreased with the dehydration treatment in both the WT and *35S:mSlARF10-6* (Table 3), and g_w (WT) < g_w (*35S:mSlARF10-6*) at 0 h of treatment. Therefore, the overexpression of *mSlARF10* decreased stomatal density, but the stomatal aperture increased. The stomatal conductance to water vapour was increased in *35S:mSlARF10-6* plants compared with in the WT. The g_w was higher in *35S:mSlARF10-6* than in the WT before 6 h of dehydration treatment, whereas g_w was lower in *35S:mSlARF10-6* after 6 h. However, the g_w and actual water loss rate were not consistent; thus, there may be another water loss pathway after dehydration treatment for 6 h. The change in the speed and extent of stomatal closure in *35S:mSlARF10-6* implied that the main factor regulating stoma ABA was different from that in the WT. The stomatal morphology further implied that *SlARF10* may play a function in stomatal development and the response to water stress by regulating the stomatal aperture.

miR160-resistant ARF10 overexpression increases ABA accumulation and sensitivity during drought

Drought stress triggers the production of the phytohormone ABA, which causes stomatal closure (Finkelstein et al. 2002) that may be induced by complex signal transduction (Schroeder et al. 2001). To verify that stomata-dependent water loss was altered in *35S:mSlARF10-6*, the endogenous ABA content and ABA-induced stomatal closures were investigated during dehydration treatment (Table 4). The accumulated endogenous ABA was gradually increased in the WT over 0–24 h of dehydration treatment. The content of ABA was also increased in *35S:mSlARF10-6* with the dehydration treatment, and reached the peak at the 6 h, then slightly decreased finally kept the high level and was always significantly higher than in the WT over 0–24 h of dehydration treatment (P < 0.01). ABA sensitivity experiments were performed to determine whether *SlARF10* is involved in ABA signalling. Non-stressed detached leaves from *35S:mSlARF10-6* and WT were perfused for 2 h in AXS + ABA solution, after which the leaves were subjected to the drought condition. ABA treatment could decrease water loss in both *35S:mSlARF10-6* and WT leaves considerably (Fig. 4). The largest decline in water loss from ABA-treated *35S:mSlARF10-6* leaves, approximately 20%, occurred after 6 h of dehydration treatment, which was even lower than the water loss from ABA-treated WT leaves before
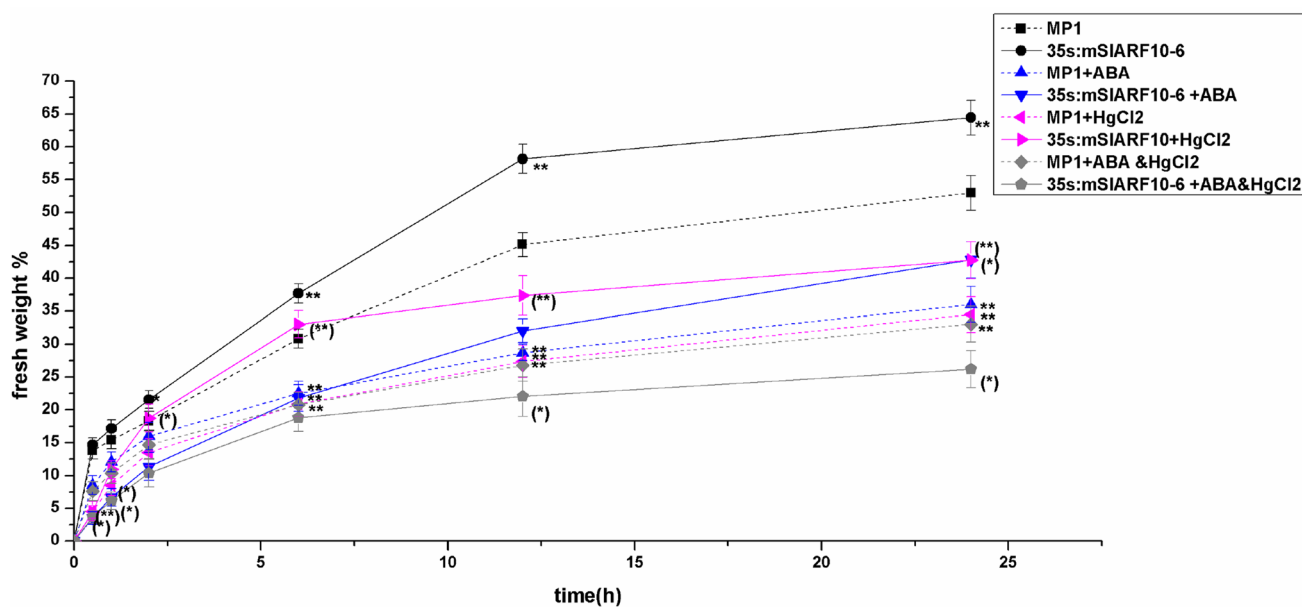

Fig. 4 Water loss percentages from the leaves of WT and *35S:mSlARF10-6* plants as well as the leaves with ABA treatment, HgCl$_2$ treatment or combined ABA and HgCl$_2$ treatment. Both WT and transgenic plants were grown under normal conditions for 6 weeks, and then, the leaves were excised from the fifth leaves of the plants.

The excised leaves were perfused for 2 h with ABA for 2 h, HgCl$_2$ or ABA and HgCl$_2$ and then weighed under the dehydration treatment. Data represent means ± SD of four independent experiments. *Indicates a significant difference (P ≤ 0.05), and **indicates a highly significant difference (P ≤ 0.01)
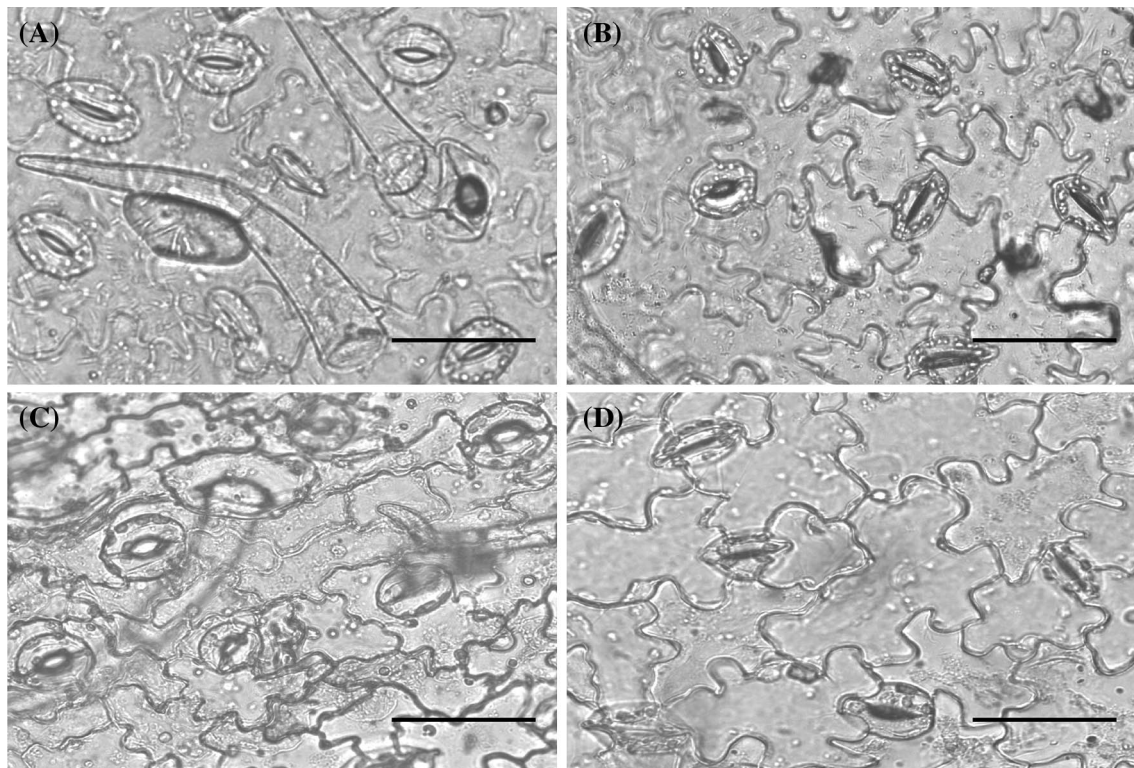
Fig. 5 WT and *35S:mSlARF10-6* leaves were treated with ABA. Stomatal aperture with the ABA treatment decreased in transgenic *35S:mSlARF10-6* leaves more notably than in WT leaves. **a** WT leaves with ABA treatment after 0 h; **b** WT leaves with ABA treatment after 6 h; **c** *35S:mSlARF10-6* leaves with ABA treatment after 0 h; **d** *35S:mSlARF10-6* leaves with ABA treatment after 6 h. Microscopic views of stomata in the fifth leaves of transgenic and WT plants. Six-week-old plants were used for all experiments. *Bar* 25 μm

6 h. The speed of water loss in *35S:mSlARF10-6* was higher than in the WT, but after treatment with ABA, it was much lower than in the WT before 6 h of dehydration treatment. This result indicated that ABA had a larger inhibitory effect on water loss in *35S:mSlARF10-6* leaves than in the WT during the early dehydration treatment. Although ABA treatment could also significantly decrease the stomata aperture size in the WT, leaves from *35S:mSlARF10-6* showed greater sensitivity to the ABA treatment and exhibited a swifter and larger decrease in stomatal aperture size (Fig. 5). Only partial stomas in the WT plants were closed after 6 h of ABA treatment, and nearly all of the stomas in *35S:mSlARF10-6* were closed after dehydration treatment for 6 h. The decrease in stomata aperture size after ABA treatment demonstrated that ABA sensitivity in *35S:mSlARF10-6* was higher than in the WT. The calculated stomata-dependent water loss in the WT was higher than in *35S:mSlARF10-6* after the dehydration treatment for 6 h. However this cannot explain why the water loss in *35S:mSlARF10-6* exceeded that in the WT after ABA treatment for 6 h. This result implies another water loss pathway besides stomata.

The advanced aquaporin activity in *35S:mSlARF10* contributes to water loss

Water movement across the leaf cell membranes is regulated by AQPs in the membranes (Heinen et al. 2009); thus, to reveal weather there is a difference in water movement in WT and *35S:mSlARF10-6*, WT and *35S:mSlARF10-6* leaves were treated with $HgCl_2$ (aquaporin inhibitor) to reveal the function of AQPs in leaf water loss (Fig. 4). The $HgCl_2$ treatment significantly inhibited water loss in the WT, and the inhibition function became gradually stronger with increasing dehydration treatment time, especially after 6 h. In *35S:mSlARF10-6*, $HgCl_2$ showed complete inhibition of water loss after 6 h of dehydration treatment, although it did play a significant role before 6 h. After combining ABA and $HgCl_2$ to treat the WT and *35S:mSlARF10-6* leaves, the water loss percentage was even significantly lower in *35S:mSlARF10-6* than in the WT (Fig. 4). From the K_{leaf}, it was also found that the water hydraulic conductance was higher in the *35S:mSlARF10-6* leaves (Fig. 2b). This suggested that the effect of AQP activity on water movement in leaves was higher in the *35S:mSlARF10-6* leaves.

ABA biosynthesis/signalling, aquaporin and stomatal development genes in *35S:mSlARF10*

Next, we conducted deep sequencing using RNA from the fifth leaves of WT and *35S:mSlARF10-6* plants to determine the effects of the *35S:mSlARF10-6* leaves on global ABA biosynthesis, signalling and AQP gene expression. We identified five AQP genes that were significantly upregulated in the *35S:mSlARF10-6* and two ABA biosynthesis and twelvesignal/response genes that were also significantly upregulated or down regulated by more than twofold (P < 0.05) in *35S:mSlARF10-6* (Table 5). One transcription factor was *SlABI5-like* (ABSCISIC ACID-INSENSITIVE 5-like), which is known to be involved in the ABA signalling pathway, and the RT-PCR results validated the transcriptome data (Fig. S2). Furthermore, some genes related to stomatal development and regulation were significantly downregulated in *35S:mSlARF10-6* (Table 5). The transcript factors *MUTE, TOO MANY MOUTHS* (*TMM*) and *LRR* receptor-like serine/threonine-protein kinase ERECTA-like were significantly lower in *35S:mSlARF10-6* than in the WT.

Promoter analysis of the upregulated expression of AQP genes in *35S:mSlARF10-6*

The above data demonstrated that the expression of five AQP genes was induced; thus, the promoter regions (2500-bp upstream sequences) of these genes were subjected to motif analysis using PlantCARE to analyse the cis-acting element in those upregulated AQP. The results show that among the AQPs, there were two upregulated AQP gene promoters that contained only AuxRE (TGTCTC/GAGACA); one AQP gene promoter contained only ABRE, whereas the other two had both ABRE and AuxRE (Table S2; Fig. S1).

The three most upregulated AQP genes (Solyc10g083880.1 '*SlTIP1-1-like*', Soly06g011350.2 '*SlPIP2;4*' and Solyc05g008080.1 '*SlNIP-type like*') were selected to verify that AuxRE and ABRE upregulated the expression of the AQP genes, and their promoters contained ABRE or both AuxRE and ABRE, respectively, which expression levels were upregulated 21.5, 5.04 and 2.7 times in *35S:mSlARF10-6*. Chimeric constructs consisting of the *SlPIP2;4* or *SlNIP-type-like* four-promoter fragments and *SlTIP1-1-like* two-promoter fragments and the GUS reporter gene (Fig. 6a) were introduced

Table 5 Differentially up-regulated AQPs, ABA and stomatal development related genes in leaves of *35S:mSlARF10-6*

Function/biological process	Ensembl ID	Annotation	Fold change
Transport	Solyc10g083880.1	Aquaporin TIP1-1-like	21.98
	Solyc06g011350.2	Plasma membrane intrinsic protein 2;4	5.04
	Solyc03g005980.2	Aquaporin NIP1-1-like	2.71
	Solyc05g008080.1	Probable aquaporin NIP-type-like	2.71
	Solyc10g054790.1	Probable aquaporin PIP1-2-like	2.30
ABA biosynthesis	Solyc11g011240.1	Geranylgeranyl pyrophosphate synthase 1	10.83
	Solyc02g085700.1	Geranylgeranyl pyrophosphate synthase 3	2.20
ABA signal/response	Solyc04g071150.2	Abscisic acid 8′-hydroxylase 3-like	4.51
	Solyc02g084930.2	Abscisic acid 8′-hydroxylase 3-like	2.42
	Solyc05g052520.2	Probable protein phosphatase 2C 30-like	2.12
	Solyc02g083420.2	Probable protein phosphatase 2C 60-like	2.26
	Solyc02g092750.2	Probable protein phosphatase 2C 78-like	2.18
	Solyc05g053290.2	Probable protein phosphatase 2C 49-like	2.90
	Solyc07g040990.2	probable protein phosphatase 2C 6-like isoform2	2.20
	Solyc12g099600.1	Probable protein phosphatase 2C 40-like	3.28
	Solyc03g007310.2	Abscisic acid receptorPYL8-like isoform 1	0.46
	Solyc03g095780.1	Abscisic acid receptor PYL4-like	0.48
	Solyc09g015380.1	Abscisic acid receptor PYL6-like	0.23
	Solyc09g009490.2	Protein ABSCISIC ACID-INSENSITIVE 5-like	2.41
Stomatal development	Solyc08g061560.2	LRR receptor-like serine/threonine-protein kinase ERECTA-like	0.101
	Solyc12g042760.1	Protein TOO MANY MOUTHS-like	0.028
	Solyc09g091760.1	Transcription factor MUTE-like	0.541

Functional categories were based on the biological/molecular functions and standardized eggNOG descriptions within the tomato database. Fold change column indicates two-fold or greater up-regulation. Fold change > 2.0

into tomato leaves. The transient expression assay demonstrated that the GUS activity of the complete *SlPIP2;4* promoter fragment was higher in *35S:mSlARF10-6* than in the control, and the activities of the GUS promoters containing only ABRE or AuxRE were both higher in *35S:mSlARF10-6* than in the WT (Fig. 6b). The GUS activity of the complete *SlNIP-like* promoter was higher in *35S:mSlARF10-6* than in the control, and the activity of GUS containing only AuxRE

was also enhanced, whereas ABRE exhibited depressive effects (Fig. 6c). The GUS activity of the promoter containing the ABRE of *SlTIP1-1-like* was higher in *35S:mSlARF10*, and the GUS activity of the promoter without the ABRE of *SlTIP1-1-like* was not changed significantly (Fig. 6d). These results implied that the upregulated expression of AQP is induced by AuxRE and sometimes by ABRE in *35S:mSlARF10-6*.

Fig. 6 The promoter fragments of Soly06g011350.2 (*SlPIP2;4*), Solyc05g008080.1 (*SlNIP-type like*), Solyc10g083880.1(*SlTIP1-1-like*) were cloned into the pBI101 plant expression vector and transformed into Agrobacterium EH105 to inject WT and *35S:mSlARF10-6* tomato leaves with the GUS reporter gene. **a** The promoter sequence analysis to illustrate the role of each element (AuxRE and ABRE), the complete promoter sequence with both AuxRE and ABRE (PIP-a, NIP-a), the promoter sequence with only AuxRE (PIP-b, NIP-b), the promoter sequence with only ABRE (PIP-c, NIP-c, TIP-a) and the promoter sequence without both AuxRE and ABRE (PIP-d, NIP-d, TIP-b) were cloned into the vector. **b** The GUS activity analysis of the promoter (PIP-a, PIP-b, PIP-c, PIP-d). **c** The GUS activity analysis of the promoter (NIP-a, NIP-b, NIP-c, NIP-d). **d** The GUS activity analysis of the promoter (TIP-a, TIP-b). *Bars* indicate the standard errors of three replicates. The control treatment is the background. *Indicates a significant difference ($P \leq 0.05$), and **indicates a highly significant difference ($P \leq 0.01$)
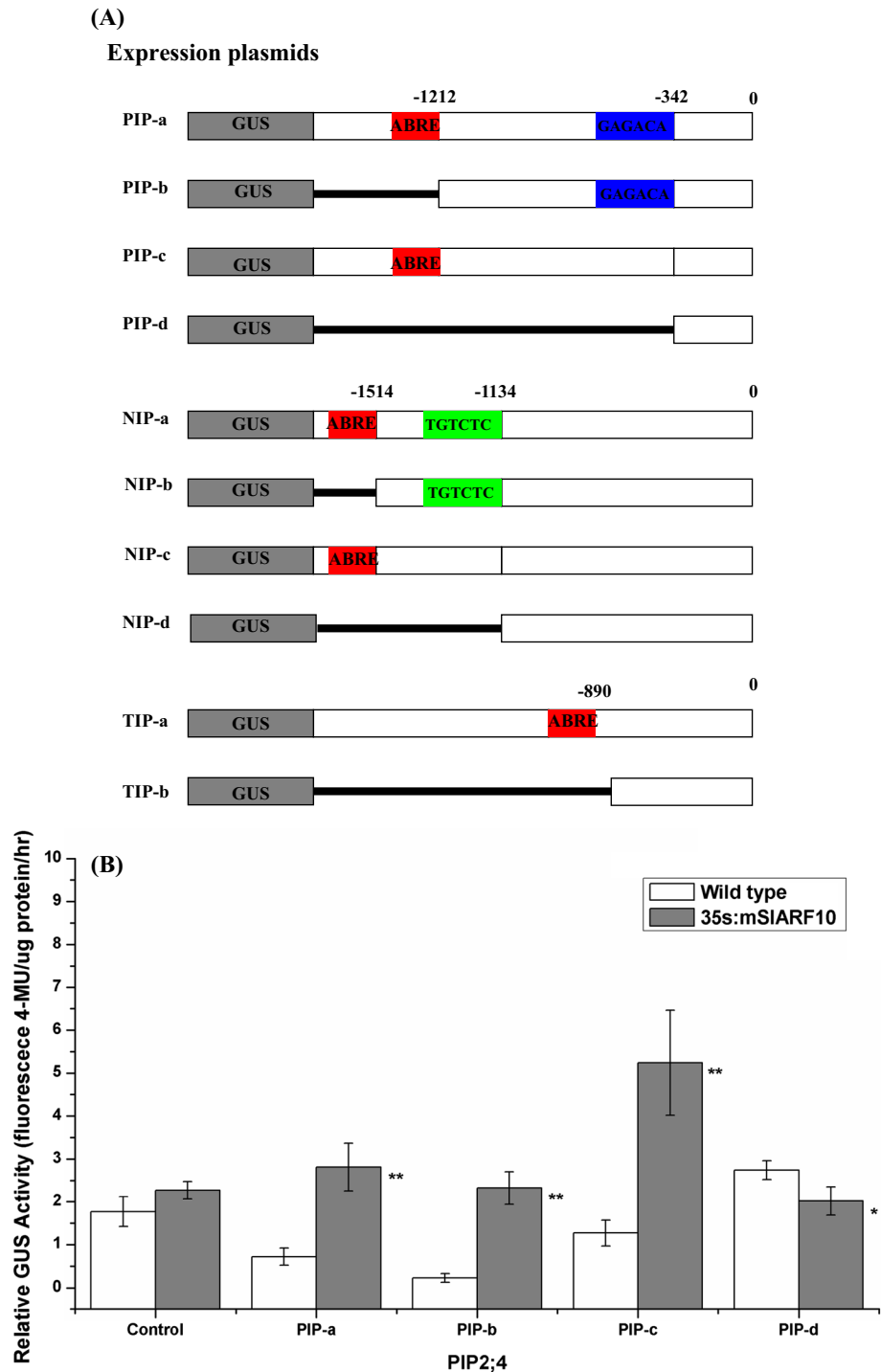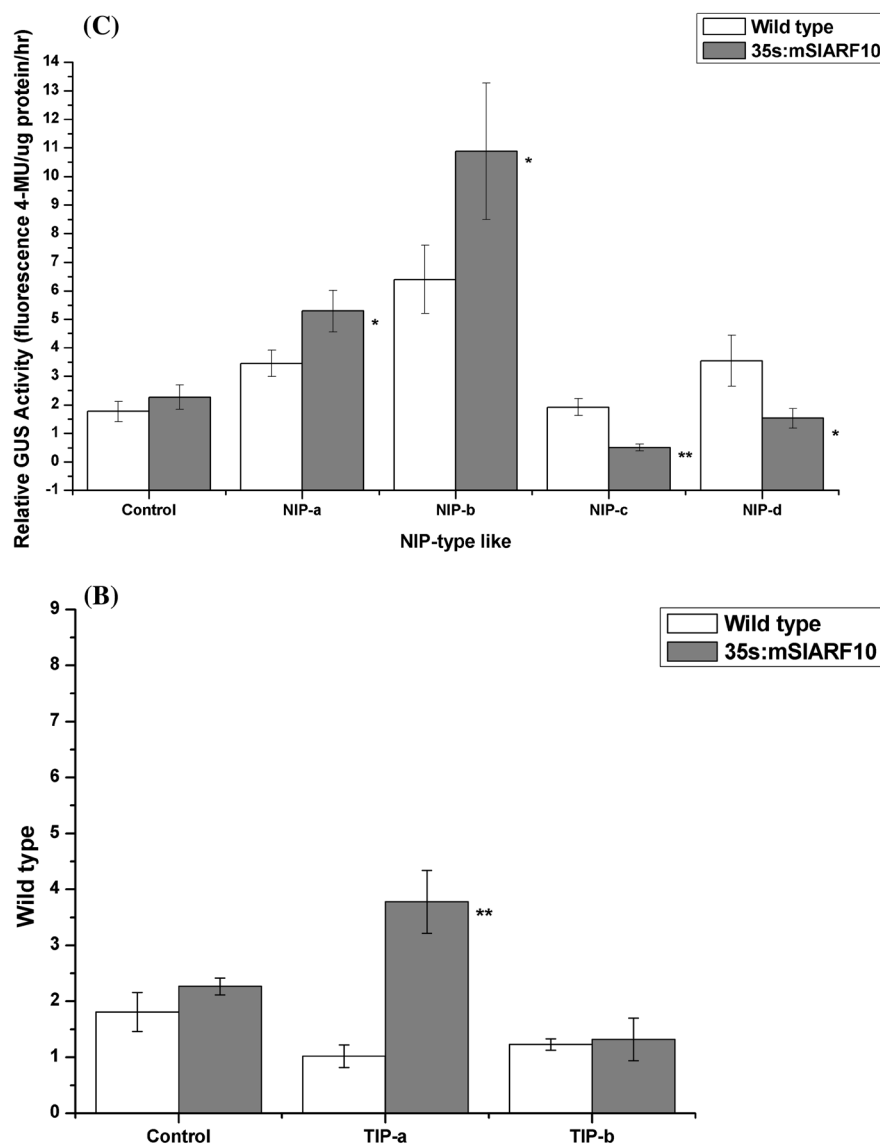
Fig. 6 continued

SlABI5-like stimulates AQP expression, and ARF10 is required to induce AQP expression

In *35S:mSlARF10-6, mSlARF10* and *ABSCISIC ACID-INSENSITIVE 5-like* (*SlABI5-like*) are two suitable candidates for the direct binding of those elements; thus, we first examined the binding of the promoter fragments of (Soly06g011350.2) '*SlPIP2;4*' and (Solyc05g008080.1) '*SlNIP-type like*' containing AuxRE to the *SlARF10* protein. At 318–368 upstream of the start codon (ATG), in Soly06g011350.2 had an AuxRE (GAGACA), and Solyc05g008080.1 had an AuxRE (TGTCTC) at 1039–1089 bp upstream of the start codon (ATG). We performed EMSAs with an in vitro-translated ARF10 protein and the 5′ promoter region of the Soly06g011350.2 and Solyc05g008080.1 with biotin labelling. We first constructed a *SlARF10* and pET30a fusion recombinant

plasmid, and after sequencing, the plasmid was transferred into the 'rosseta' bacterial strain for expression. A *SlARF10* fusion His recombinant protein was obtained by induction and purification, and the protein, which was purified by Coomassie Brilliant Blue staining, was approximately 72 kDa in size (Fig. 7a). The size was approximately the sum of ARF10 (63 kDa) and His (6 kDa) and verified by His antibody Western blotting, which indicated that this protein was indeed the His-ARF10 fusion protein. An EMSA assay indicated that the His-ARF10 protein bound the oligo DNA with molecules with the *SlPIP2;4* and *SlNIP-type* promoter fragments, respectively (Fig. 7b), which suggests that *SlARF10* could bind the promoter with TGTCTC or GAGACA to regulate the expression of the *SlPIP2;4* and *SlNIP-type like* genes.

A Y1H assay was also performed to test the association of *SlARF10* with the promoter of the *SlPIP2;4* and *SlNIP-type*

(A)

(B)

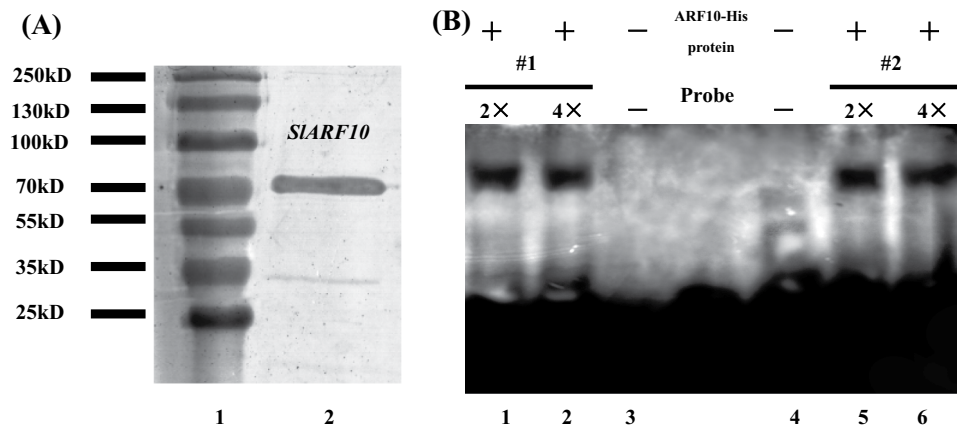Fig. 7 **a** In vitro-translated SlARF10 analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. *Lane 1* protein markers. *Lane 2 SlARF10*-His fusion protein. **b** EMSA of in vitro-translated SlARF10 protein binding to the 318–368 fragment of Soly06g011350.2 (PIP2;4) promoter (GACACA) and the 1039–1089 fragment of Solyc05g008080.1 (NIP-type like) promoter (TGTCTC). #1 probe is Soly06g011350.2 (*SlPIP2;4*) promoter; #2 probe is Solyc05g008080.1 (*SlNIP-type like*) promoter. *Lane 1* control; *lane 2* #1 probe two times volume; *lane 3* #1 probe four times volume; *lane 4* control; *lane 5* #2 probe two times volume; *lane 6* #2 probe four times volume

like genes. The results showed that *SlARF10* could associate with two AuxRE located at −6 bp to −1399 bp of the *SlPIP2;4* and −7 bp to 1466 bp of the *SlNIP-type like* genes, respectively (Fig. 8), and the mutation of these binding sites could break this association, supporting the hypothesis that *SlARF10* was the transcription factor associated with the AQPs. Next, further confirming the effect of the ABA signal on the AQP genes, Solyc09g009490.2 (*ABSCISIC ACID-INSENSITIVE 5-like*) was upregulated by two fold in *35S:mSlARF10-6* from the transcriptome. We chose the AQP gene (Solyc10g083880.1, *SlTIP1-1-like*), whose expression levels were upregulated 21 times from the transcriptome data, and the *SlNIP-type like*, which was upregulated 2.7 times, for the ABRE binding test because they could show enhanced GUS activity in transient expression. As shown in Fig. 8b, *SlABI5-like* bound to the ABRE containing DNA fragments (GAGACA) of the *SlTIP1-1-like* promoter but failed to bind to ABRE-mutated motifs (AAAAAA), and the same result was obtained for the association with *SlNIP-type-like* gene. The results demonstrated that upregulated AQP genes could also be directly stimulated by *SlABI5-like*.

The effect of transient expression of *SlARF10* and *SlABI5* on leaf water loss

We have shown that *SlABI5* and *SlARF10* bind on the AQPs promoters. For further confirmation of roles of *SlABI5* and *SlARF10* in leaf water loss, *SlABI5* overexpression and *SlARF10* silencing vectors were transiently overexpressed in tomato leaves in an Agrobacterium mediated process. Three days after the injection, the expression levels of *SlABI5* and *SlARF10* were confirmed. The results showed that the expression levels of *SlARF10* in containing pB7GWIWG2(II)-ARF10 tomato leaves were decreased more than three times

(Fig. S9). Furthermore, the expression levels of *SlABI5* were increased more than five times in the tomato leaves containing pBI121-ABI5 (Fig. S9). Next we performed a water loss experiment with the transient expression leaves. The results showed that transient silencing of *SlARF10* significantly decreased the water loss rate compared to CK especially after the 6 h dehydration treatment (Fig. 9a), and the expression levels of the most of the AQP family members were decreased, especially Solyc10g083880.1 '*SlTIP1-1-like*' and Solyc05g008080.1 '*SlNIP-type-like*' (Fig. S9). The transient overexpression of *SlABI5* showed that the water loss rate was significantly lower than CK before the 6h dehydration treatment,but after 6h, the water loss rate was almost the same as CK (Fig. 9b). The expression levels of some of the AQP family members were increased especially Solyc10g083880.1 '*SlTIP1-1-like*' and Solyc05g008080.1 '*SlNIP-type like*' whose promoters were confirmed to be bound by *SlABI5* through a Y1H assay (Fig. S9), but the expression levels of other AQP family members were largely not increased. It was also found that the AQP genes with the greatest increases in expression were those whose promoters contained ABRE elements (Fig. S10).

AQP expression is tissue specific in the upper epidermis of the tomato leaf

In situ hybridization experiments were performed to further reveal the distribution and accumulation of up-expression of AQP mRNA in *35S:mSlARF10-6*. *SlPIP2;4* (Solyc06g011350.2) was selected because its promoter contains both AuxRE and ABRE and is the second most highly up-expressed AQP in *35S:mSlARF10-6*. A single-stranded RNA complement to the *SlPIP2;4* gene produced a weak signal that was mainly observed in the leaf epidermal
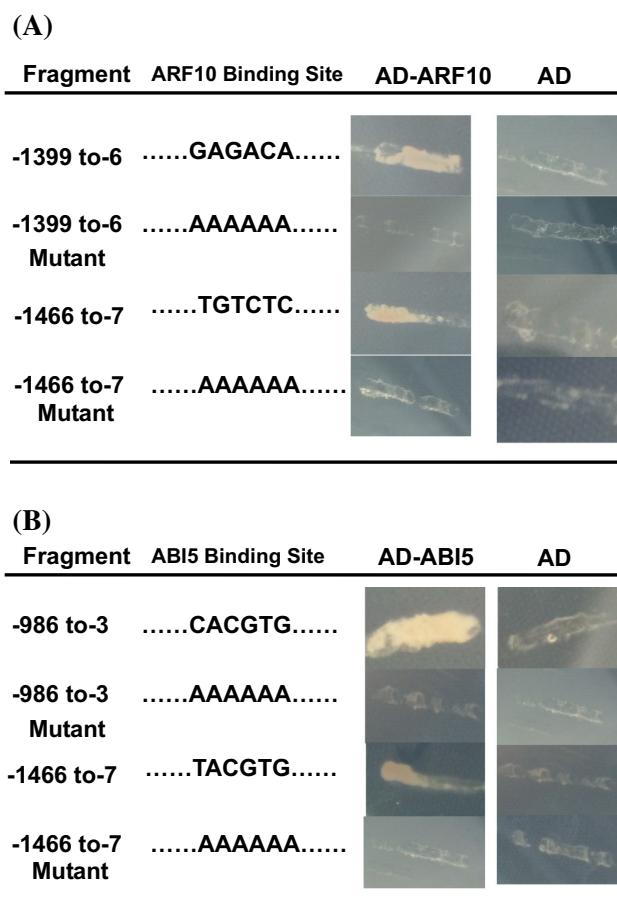
(A)

Fragment	ARF10 Binding Site	AD-ARF10	AD
-1399 to-6	......GAGACA......		
-1399 to-6 Mutant	......AAAAAA......		
-1466 to-7	......TGTCTC......		
-1466 to-7 Mutant	......AAAAAA......		

(B)

Fragment	ABI5 Binding Site	AD-ABI5	AD
-986 to-3	......CACGTG......		
-986 to-3 Mutant	......AAAAAA......		
-1466 to-7	......TACGTG......		
-1466 to-7 Mutant	......AAAAAA......		

Fig. 8 **a** The association of *SlABI5-like* with the promoter of Soly06g011350.2 '*SlPIP2;4*' and Solyc10g083880.1 '*SlTIP1-1-like*' by a Y1H assay. **b** The association of SlARF10 with the promoter of Soly06g011350.2 '*SlPIP2;4*', Solyc05g008080.1 '*SlNIP-type like*'. Four AQP gene promoter elements were mutated and interacted with *SlABI5-like* and *SlARF10*. Each fragment of the AQP gene promoter was ligated to a pAbAi vector to generate pAbAi-bait plasmids, and AD-ABI5-like or AD-ARF10 was transformed into the Y1HGold strain holding pAbAi-bait and screened again on a plate with selective synthetic dextrose medium lacking leucyl/AbA. An empty pGADT7 vector (AD) was transformed as a negative control

cells and neighbouring midrib mesophyll cells in the WT (Fig. 10b). The probe produced stronger chromogenic signals in 35S:mSlARF10-6 leaves from the epidermis to midrib cells (Fig. 10c), and greater *SlPIP2;4* expression was detected in 35S:mSlARF10-6 leaf epidermis than in the WT.

SlARF10 links both auxin and ABA signalling in the regulatory mechanism to mediate leaf water loss

The results of this study could be used to build a network to illustrate the precise role of *miR160* in the regulation of *ARF10* in plant water stress (Fig. 11). Thus, we proposed a model of the effect of the ABA-auxin interaction on the control of water loss. When auxin signalling was activated, Aux/IAA was degraded, and ARF10 was freed at a high

transcription level. Although ARF10 could enhance ABA content through active ABA synthesis gene expression and promote the ABA signal through ABI5 expression, which resulted in stomatal closure under drought. *SlARF10* overdose enhanced the leaf water loss rate by modifying stomatal development and AQP expression. And *SlABI5* increased the AQPs which induced the cell osmotic in leaf. The rational ARF10 expression levels mediated by *miRNA160* may play an important role in maintaining leaf water balance during leaf development and adaptation to water stress.

Discussion

Overexpression of *mSlARF10* resulted in narrower leaves but a higher rate of water loss

An earlier article showed that overexpression of *mSlARF10* could alter leaf size to result in leaflets with extremely narrow blades (Hendelman et al. 2012). Narrower leaves typically have lower water loss rates due to the low transpiration of the small leaf area and are selected during breeding as a trait to enhance drought resistance (Liu et al. 2010). In 35S:mSlARF10 tomatoes, the overexpression of mSlARF10 resulted in greater water loss from the narrower leaves; however, 35S:SlARF10 which also had narrow leaves, demonstrated a lower rate of water loss than 35S:mSlARF10-6, and a significantly higher rate of water loss than WT (Fig. S6). A reasonable explanation for this result is that the expression levels of *SlARF10* were lower than *35S:mSlARF10-6* but higher than WT. Thus, the regulation of *miR160* on *SlARF10* maintains the balance of the expression levels of *ARF10* to mediate the leaf water loss rate. There is higher water loss efficiency in *35S:mSlARF10* tomato leaves than in the WT. The *35S:mSlARF10* tomato has a higher K_{leaf} than the WT, which supports this hypothesis.

The leaf is a hydraulic bottleneck that accounts for a large part of plant resistance to drought (Brodribb and Cochard 2009), and water loss is restricted by the hydraulic and stomatal conductance of the leaf (Pou et al. 2013). The leaf hydraulic conductance (K_{leaf}) is determined by the multiple components of the water transport pathways; water moves through the petiole and vein xylem, as well as bundle sheath and mesophyll tissue, before evaporating through the stomata. The decline in K_{leaf} may be caused by losses in conductivity in one or more of these components (Johnson et al. 2009; Scoffoni et al. 2011), and among them, stomatal closure is the major regulator of plant water loss. When stomata open for photosynthesis, water is lost via transpiration, and K_{leaf} must remain high to prevent the tissue water potential from declining sufficiently far to trigger a decline in stomatal conductance (Tyree and Zimmermann 2002).
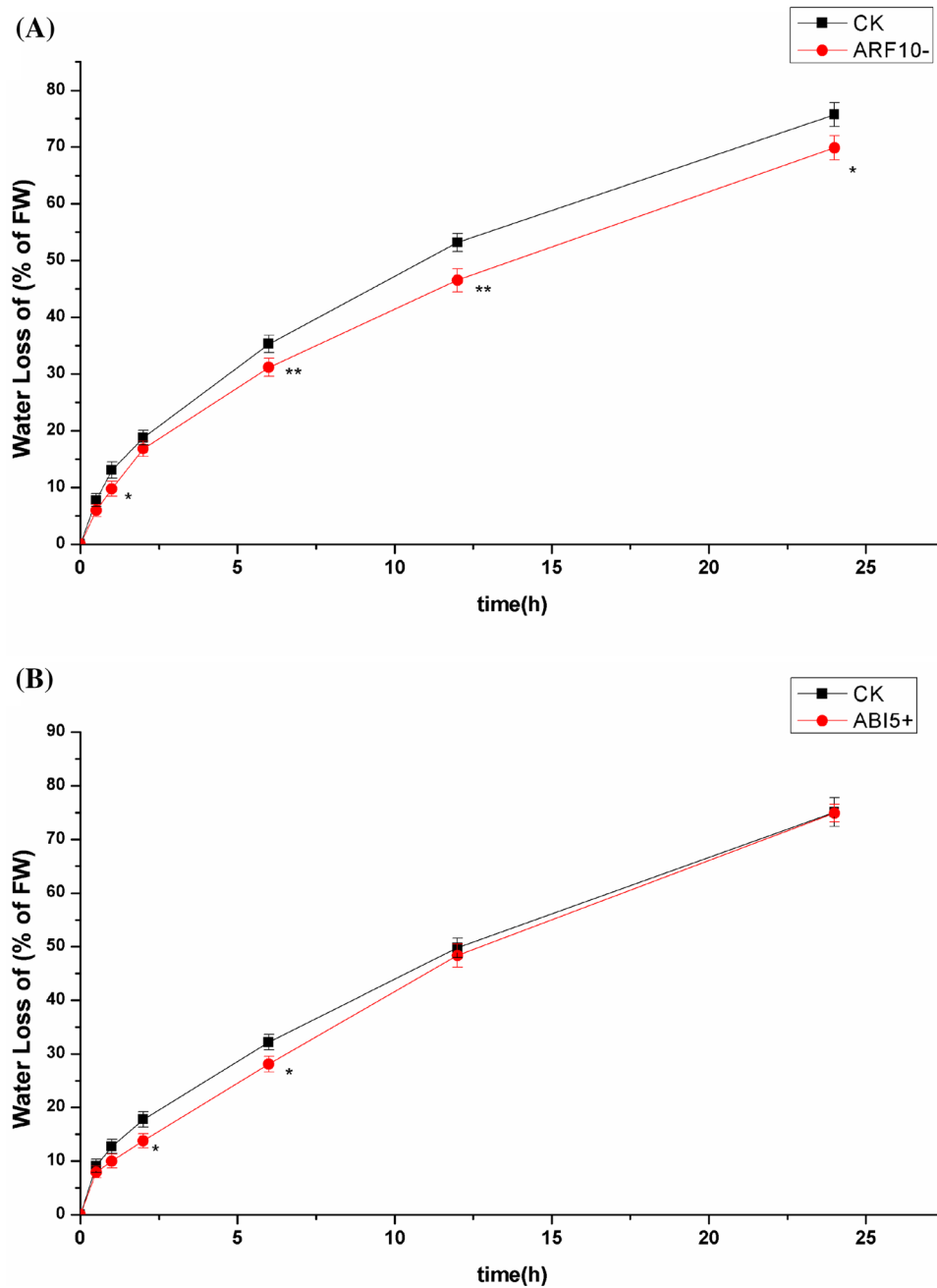
Fig. 9 Water loss percentages from CK leaves and transient expression leaves containing pB7GWIWG2(II).0-ARF10 and pBI121-ABI5. **a** The water loss of leaves that were injected with the *Agrobacterium* harbouring pB7GWIWG2(II).0-ARF10 or pB7GWIWG2(II).0. **b** The water loss of leaves that were injected with the *Agrobacterium* harbouring pBI121-ABI5 and pBI121. The excised leaves were bench-dried and weighed. *Indicates a significant difference (P ≤ 0.05), and **indicates a highly significant difference (P ≤ 0.01)

However, stomatal closure may be decreased under water stress in response to a decline in leaf hydraulic conductance (Sack and Holbrook 2006). Overexpression of *mSlARF10* resulted in a larger individual stoma size but lower stomatal density in the leaf, and the transcriptome data demonstrated that the transcript factors regulating stomatal development were decreased significantly in *35S:mSlARF10-6*. In *35S:SlARF10*, the stomatal

parameters were between the *35S:mSlARF10-6* and WT plants and did not obviously change (Table S3; Fig. S7). *ARF10* regulated by *miR160* was also important for the regulation of stoma development. The overexpression of *MUTE* has been implicated in changing leaf epidermis cells into stomata (Pillitteri et al. 2007; Serna 2007), and the leucine-rich repeat domain (*LRR*) receptor-like protein and *TMM* have been reported to be involved in
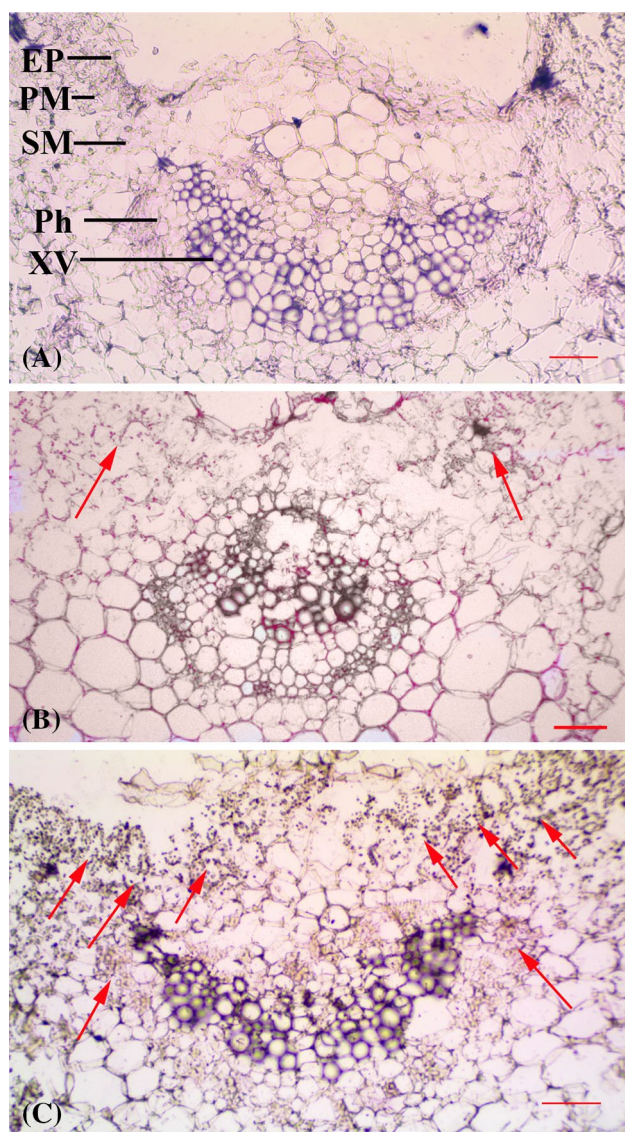
Fig. 10 Induction of Soly06g011350.2 'SlPIP2;4' in 35S:mSlARF10-6 in the leaf epidermis. Detection of SlPIP2;4 expression in leaves. a Control cross-sections probed with a SlPIP2;4 sense probe; **b** the cross-sections of wild type leaves with a DIG-labelled ssRNA probe complementary to a SlPIP2;4 probe; **c** the cross-sections of 35S:mSlARF10-6 leaves with a DIG-labelled ssRNA probe complementary to SlPIP2;4. Bar 50 μm

stomatal regulation (Hunt and Gray 2009). Theoretical water loss based on calculated stomatal-dependent water loss was confirmed to be consistent with the actual water loss in 35S:mSlARF10 and the WT under normal conditions. However, the water loss rate does not precisely adhere to the calculated stomatal-dependent water loss in 35S:mSlARF10 and the WT after water stress for 6 h, which indicates that there may be a complex water loss pathway in 35S:mSlARF10-6.

In contrast, reducing AQP activity and potentially emptying the water-filled cell wall pores in the bundle sheath and mesophyll is another important contributor to the decline

in K_{leaf} (Pieruschka et al. 2010; Shatil-Cohen et al. 2011). AQPs have been implicated in the determination of internal conductance to water in the leaf (Heinen et al. 2009), and reduced/enhanced AQP activity is associated with AQP activation and/or expression inhibition (Cochard et al. 2007; Nardini et al. 2005; Voicu et al. 2008, 2009). The downregulation of AQPs under water stress and coordination with stomatal conductance represent a mechanism for the coregulation of water loss at the cellular level (Pou et al. 2013).

Differences in water loss between 35S:mSlARF10 and the WT that occur when plants are treated with ABA (stomatal opening inhibitor) and $HgCl_2$ (special AQP inhibitor) further support the conclusion that stomatal closure and AQP activity contribute more to water loss in 35S:mSlARF10 than in the WT. Treatment with only ABA for less than 6 h significantly decreased water loss in 35S:mSlARF10 to an even greater degree than in the WT treated with ABA, which indicated that 35S:mSlARF10 has greater water loss efficiency through the stomata. In the next 18 h, 35S:mSlARF10 treated with ABA exhibited a lower water loss rate than WT, revealing that the stomata are not the only reason for the more rapid water loss in 35S:mSlARF10. Using a special AQP inhibitor, $HgCl_2$, to treat the leaf had little effect before 6 h, but it significantly inhibited the water loss in 35S:mSlARF10 after 6 h. Furthermore, in potatoes, a significant decrease in stomatal conductance may occur at an early stage of drying, even before a significant decrease in leaf water potential (Jefferies and Mackerron 1989). Combining the ABA and $HgCl_2$ treatments could further depress water loss in 35S:mSlARF10; it was even lower than that in treated WT throughout the duration of water stress. These results reveal that the overexpression of mSlARF10 could alter leaf water loss by modifying stomatal closure and AQP activity during drought. We also examined the influence of ABA or $HgCl_2$ treatments on 35S:SlARF10 leaf water loss (Fig. S8). Both demonstrated little inhibition of the water loss in 35S:SlARF10 leaves. The degree of inhibition in 35S:SlARF10 was between the 35S:mSlARF10-6 and MP1 by ABA or $HgCl_2$.

Overexpression of mSlARF10 affects ABA synthesis and signalling to regulate stomatal closure

The plant hormone ABA accumulates under water deficit conditions and plays a major role in dehydration response and tolerance (Wan et al. 2004). A high ABA concentration around guard cell results in stomata closure, and the expression of AQPs and the depression of their activities help plants conserve water. In 35S:mSlARF10, high ABA content and sensitivity increase stomatal closure and the response to ABA. In ABA biosynthesis, analysis of RNA sequences did not reveal differences in the expression of key ABA biosynthesis genes (NCED) but did identify
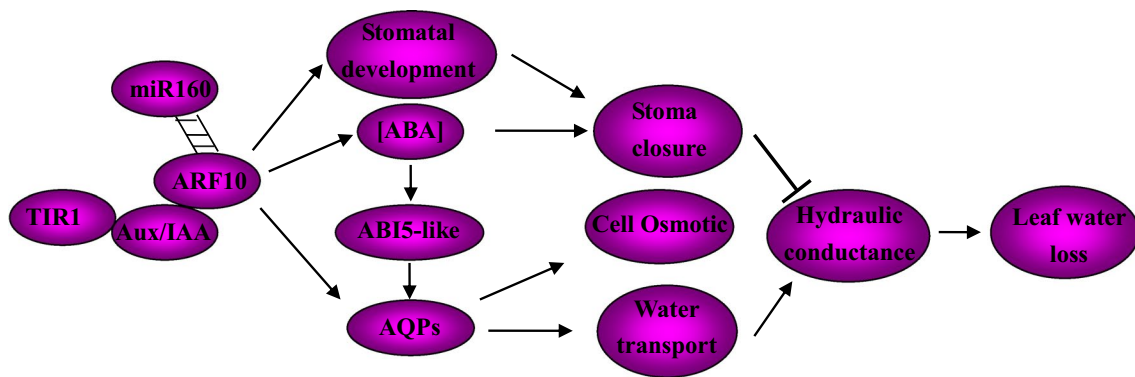
Fig. 11 A proposed model for the role of miR160-regulated ARF10 in response to leaf water loss. When auxin signalling was activated, AUX/IAA degraded, and ARF10 was freed at a high transcription level. miR160 also regulates the expression levels of ARF10, which directly influences stomatal development. ARF10 increases ABA content and promotes ABA signal 'ABI5-like' expression, resulting in sensitive stomatal closure under drought. ARF10 increased the leaf water loss rate by directly increasing AQP expression to enhance the hydraulic conductance. Stomatal-dependent and AQP-dependent hydraulic conductance both influence the water loss in the leaf. → and — indicate positive and negative modes of regulation, respectively; [ABA] indicates ABA concentration

differential expression of geranylgeranyl pyrophosphate synthase, which is a key enzyme in the carotenoid biosynthetic pathway, with significant up-regulation in *35S:mSlARF10*. Two pathways have been proposed for the biosynthesis of ABA. It has been shown that the ABA produced from the oxidative cleavage of carotenoids is the main pathway (Seo and Koshiba 2002). In this pathway, IPP is converted to a C20 product by geranylgeranyl pyrophosphate (GGPP). It is known that GGPP is a precursor of carotenoids (Cunningham et al. 1993). This procession is also reported to be involved in plant responses to the abiostresses such as salt stress and drought stress (Ruiz-Sola et al. 2014). During dehydration, the plant ABA content would show a continues increased trend to improve the adaption (Seo and Koshiba 2002). In this study, the ABA content of *35S:mSlARF10-6* with dehydration treatment have a maximum at 12 h and then decrease but maintain a high level. The reasonable explanation is that the ABA content reached highest level at 50 % relative water content during dehydration (Di Blasi et al. 1998). In our data, the *35S:mSlARF10-6* relative water content at 12 h dehydration treatment is around 50 %, even the longer treatment would make the *35S:mSlARF10-6* relative water content smaller, the ABA accumulation is not increased. In addition to the ABA content, the ABA signal is also reportedly involved in the adaptive responses of plants to drought stress. There are three core components: the ABA receptors PYR/PYL/RCARs (hereafter the 'PYLs'), the negative regulators named PP2Cs (type 2C protein phosphatases), and positive regulators termed SnRK2s (subfamily 2 of SNF1-related kinases) (Ma et al. 2009; Park et al. 2009). The expression of the CYP707A gene encoding ABA 8′-hydroxlase could increase in response to dehydration stresses as well as high ABA levels (Saito et al. 2004). Water stress alters the ABA signal by modifying the core gene expression, and

expression analysis indicated that most *SlPYL* genes were downregulated, whereas most *SlPP2C* and *SlSnRK2* genes were upregulated by dehydration in tomato leaf (Sun et al. 2011). ABI3 is sufficient to change ABA responsiveness in vegetative tissues, and ABI1 is required for ABI3-regulated processes (Bonetta and McCourt 1998). The altered ABA signal would modify the ABA response during water stress, and overexpression of *miR160* resulted in reduced sensitivity to ABA during germination. Transcriptome analysis of germinating mARF10 seeds indicated that the typical ABA-responsive genes expressed during seed maturation were overexpressed in germinating mARF10 seeds (Liu et al. 2007), and further study has revealed that the highly similar ARF10 and ARF16 proteins could both control the expression of *ABI3* during seed germination but without directly binding to the *ABI3* promoter (Liu et al. 2013).

In *35S:mSlARF10* leaves, both the ABA content and the ABA sensitivity (stomatal closure rate response to exogenous ABA) were significantly enhanced. RNA sequencing showed that two ABA synthesis genes and twelve ABA response/signal transduction genes containing ABI5 were significantly changed in *35S:mSlARF10* leaves, and this could indicate why ABA content and sensitivity are enhanced in *35S:mSlARF10*. It has been reported that *HvABI5* is indispensable for ABA induction of gene expression, which is an *ABI5* orthologue in barley (Zhou et al. 2013). In addition to modulating the adaptive stress response, *OsABI5* also plays crucial roles in plant fertility (Zou et al. 2007, 2008), and the rice ABI5-like gene has been shown to be an important node in the cross-talk of ABA–auxin signalling (Yang et al. 2011). However, *ARF10* may not directly bind to the *ABI5* promoter because it lacks the typical AuxRE element, but *ABI5* promoter-containing ABRE elements and high ABA content and signal exits in 35S:*mSlARF10* imply that *ARF10* could be a positive *ABI5* regulator via an indirect effect.

High leaf water loss by overexpression of *mSlARF10* might be mediated directly by *ARF10* function and not through *ABI5*

Overexpressed *mSlARF10* acts as a transcription activator to directly induce the expression of AQPs to increase the hydraulic conductance. The ARFs regulate the expression of a large set of auxin-responsive genes by binding to the auxin response elements (AuxREs) in their promoters (Guilfoyle and Hagen 2007), and the ARF protein sequences contained in the middle regions function as activation or repression domains (Ulmasov et al. 1999). There is a conflict in the literature over *SlARF10* transcription activity; Wu et al. (2011) consider it to be a repressor, whereas Kumar et al. (2011) classify it as an activator. Alternatively, the ARFs may activate or repress transcription in selected cell types that contain a different set of coactivators or corepressors (Ulmasov et al. 1999). Promoter analysis showed that the significantly upregulated AQP genes in *35S:mSlARF10* contain AuxRE, and transient expression confirmed that over-expression of *mSlARF10* could induce the promoter GUS activity. The Y1H assay and EMSA demonstrated direct binding between the *SlARF10* protein and DNA sequence containing AuxRE. Additionally, the 'ARF10-' transient expression demonstrated that the water loss rate of leaves was decreased, and most of the AQP gene expression levels were decreased. Furthermore, in *35S:mSlARF10-6*, over-expression of *mSlARF10* always induce es GUS promoter activity for AQPs promoters containeing AuxRE. However, 'ABI5+' transient expression indicated that the *ABI5* did not induce the increased leaf water loss, but induce some AQP genes expression levels increased.

AQP expression has been described in all of the leaf tissues in which high transmembrane water movement is required; however, AQPs can still facilitate other functions. During water stress, most plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs) show reduced expression (Alexandersson et al. 2005), and dehydration treatment rapidly decreases levels of *PIP1;5, PIP2;2, PIP2;3* and *PIP2;6* transcripts to one-tenth of their normal levels (Jang et al. 2004). Furthermore, post-translational regulation of PIPs has been implicated in reduced water transport under water deficit conditions. Finally, there is a negative correlation between AQP abundance and transpiration rate (Morillon and Chrispeels 2001). Plant AQPs may divide into two "classes" during dehydration. The first would be primarily involved in the maintenance of cellular water (osmotic) status, not the stress response mechanisms, and the second class of AQPs would be specifically expressed and/or regulated in the appropriate organs following stresses to compensate for the altered water potential (stress-responsive isoforms). Mutation of these isoforms would markedly alter the overall resistance of the plants

to these stresses (Hachez et al. 2006a), and this information implies that the expression of only some special AQPs is related to water loss. The responses of AQPs to water stress may involve either the up- or downregulation of gene expression or no change (Tyerman et al. 2002).

It is reasonably deduced that AQPs directly regulated by *SlARF10* play the major role in responding to water stress, while AQPs regulated by *SlABI5* may have roles in maintaining the cell osmotic balance for plants to better adopt to the environment, e.g. stomatal closure to avoid excessive water loss (Raschke 1987; Zeevaart and Yang 2005). Other reports also indicated that high expression of *ABI5* is coupled with enhanced adaptation to stress. *ABI5* expression is highest in young seedlings exposed to dehydrating stress (Finkelstein and Lynch 2000; Lopez-Molina et al. 2001). Furthermore, *ABI5* overexpressing plants are hypersensitive to abscisic acid and retain water more efficiently than WT plants (Lopez-Molina et al. 2001). The similar strategy identified for ABA can be involved in triggering the expression of AQP-like genes to achieve development and drought response (Ingram and Bartels 1996).

Overexpression of *mSlARF10* induced AQP expression in the leaf epidermis

Plant aquaporins localize in all subcellular compartments forming or derived from the secretory pathway. Special expression of a PIP and a NIP homolog on the distal side of root exo- and endodermal cells has been described in maize and rice, respectively. Such cell polarization is consistent with the uptake and centripetal transport of water. (Hachez et al. 2006a; Ma et al. 2006; Maurel et al. 2008). Our in situ hybridization results demonstrated that the abnormal upregulated AQPs in *35S:mSlARF10* were mainly expressed in the epidermis of the leaves, and the AQPs at this position might constitute a plausible reason for high water conductance and loss in *35S:mSlARF10* plants.

miR160-regulated *SlARF10* mediated auxin and ABA signalling in the leaf in response to water loss

During normal leaf development in wheat, the water loss rate of the excised leaf first increased and then decreased with increasing leaf age (Zhao et al. 2009; Yang et al. 2002). The increase in the residual transpiration rate with leaf age is due to the duration of exposure to the rigors of the external environment (Clarke and Richards 1988). Direct evidence for the important role of AQPs in leaf growth was provided by (Uehlein et al. 2003); in tobacco, *NtAQP1* overexpression increased the leaf growth rate, an effect that can be attributed to *NtAQP1* acting as both a water channel and CO_2 transporter. The expression levels of *ARF10* and *miR160* were investigated in the different

leaf development processes (Fig. S4); *ARF10* first increased in the tender, mature, old leaves and then decreased in the yellow leaves. *miR160* expression levels first decreased and then increased, which is contrary to *ARF10* expression panters. It is reasonably deduced that *ARF10*/*miR160* are involved in leaf development via coordination of water metabolism. Moreover, *miR160* and *ARF10* are involved in mediating auxin sensitivity. In *Arabidopsis, ARF10* mediates the link between ABA and auxin responsiveness during and after seed germination (Liu et al. 2007). In soybean, ectopic expression of *miR160* results in auxin hypersensitivity while *miR160* appears to promote auxin activity by suppressing the levels of the *ARF10/16/17* family of repressor ARF transcription factors (Nizampatnam et al. 2015; Turner et al. 2013). *ARF10* expression is typically highly mediated by *miR160*, and in the overexpressed *ARF10* line, the upregulated expression of *miR160* was also observed to result in cleavage of *ARF10* to maintain stable expression level (Hendelman et al. 2012). This fine tuning mechanism plays an important role in auxin sensitivity.This miRNA-mediated auxin signalling in the regulation of leaf water loss is likely an evolutionary mechanism that prevents the auxin response factor from inducing leaf water loss to maintain normal development, and it could also be crucial for improving drought stress tolerance. There are many studies indicating that miRNAs regulate ARFs to influence leaf and root development to affect drought stress and tolerance (Ding et al. 2013). The functions of *ARF10* in leaf water loss may have an evolutionary benefit. ARF10 was observed to act as a positive regulator of AQP-related genes and to contribute to leaf water loss in tomato, which elucidates a crucial mechanistic role of auxin in this well-studied biological process.

In addition to its higher degree of water loss in leaves, *35S:mSlARF10-6* may be less resistant to drought. The rosseta strain containing the SlARF10-expressed protein showed lower growth than the control on the PEG-treated plate (Fig. S3). An earlier report found slower growth and developmental defects in young Arabidopsis mARF10 seedlings, which did not survive when directly grown in soil (Liu et al. 2007); thus, we assumed that an ARF10-mediated water loss mechanism may also exist in Arabidopsis. In Arabidopsis, *AtARF10* and *AtARF16* function as the activators of *ABI3* transcription to positively regulate the ABA signal pathway, which contributes to the emerging map of the integration of hormone signalling in plant development and environmental adaption. In this study, we uncovered a previously unrecognized role of auxin in leaf water loss, namely, the promotion of leaf hydraulic and stomatal conductance through *SlARF10*. Therefore, our study establishes a molecular link between two important hormone pathways to water stress adaptation. *miR160* would regulate *ARF10* to

maintain the normal leaf development and senescence process through the regulation of water status.

Author contributions X. Liu, T. Xu and T. Li designed the research; X. Liu, X. Dong and Z. Liu performed experiments; X. Liu, Z. Shi, Y. Jiang and M. Qi analysed data; X. Liu and T. Xu wrote the manuscript.

Compliance with ethical standards

Conflict of interest We declare that we do not have any commercial or associative interests that represent a conflict of interest in connection with the work submitted.

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