

# Guard-cell-specific expression of *Arabidopsis ABF4* improves drought tolerance of tomato and tobacco

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**Abstract** Abscisic acid (ABA), the hormone associated with seed maturation and stress responses, activates a signal transduction pathway culminating in stomatal closure. Reflecting the central position of this hormone in many plant processes, many genes involved in ABA signaling have been identified. However, biotechnological approaches manipulating these genes in guard cells have rarely been applied to crops with an objective of engineering drought tolerance. We have used the *Arabidopsis* bZIP transcription factor, ABF4, under the control of guard-cell-specific *KST1* promoter for transformation of tobacco and tomato. Expression was shown to be guard-cell-specific, and ABF4-mGFP fusion proteins accumulated in guard cell nuclei. All transgenic lines showed significant increase in drought tolerance when seedlings or mature plants were subjected to prolonged water deficit. These plants exhibited significantly lower water loss per unit leaf area compared to wild type, higher water potential, and relative water contents during water deficit. Importantly, the plants did not develop the severe growth retardation frequently encountered when stress-related transcription factors were expressed constitutively, demonstrating the advantage of tissue- or cell-specific gene manipulations

as useful tools to improve plant drought tolerance and also other water-related traits under water-limiting environments.

**Keywords** Guard cell · Water deficit · Transpiration · Relative water content · Water potential

## Introduction

During their life cycle, plants are subjected to multiple environmental stresses such as water deficit, low temperature, flooding, or high salinity. Therefore, the ability of plants to adjust to adverse conditions in a timely fashion is critical for maintenance of productivity as well as for survival in prolonged inadequate conditions. Among environmental stresses, drought is one of the most devastating constraints for agriculture. Due to drought, nearly 40% loss of maize yield is estimated in North America alone (Boyer 1982). Because drought is one of the major global environmental bottleneck for agriculture, enhancing drought tolerance has immense economic impact (Umezawa et al. 2006).

Drought triggers various plant responses including the accumulation of the phytohormone ABA. ABA is a pivotal player by reducing water loss by inducing stomatal closure through changing the expression of many stress-responsive genes including the family of ABRE-binding factors/ABRE-binding bZIP proteins, ABFs/AREBs. Many ABA responsive genes feature consensus *cis*-acting elements known as ABA-responsive elements (ABREs) in their regulatory regions (Guiltinan et al. 1990;

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Yamaguchi-Shinozaki and Shinozaki 1994), and the most abundant ABREs contain the C/TACGTGGC sequence (Kang et al. 2002). Further detailed promoter analyses of ABA-responsive genes revealed that multiple ABREs or the combination of ABRE and coupling element (CE) are required for the expression (Narusaka et al. 2003; Yoshida et al. 2010). These ABA-responsive genes are upregulated by the binding of transcription factors such as ABFs/AREBs (Choi et al. 2000; Kang et al. 2002; Uno et al. 2000). *ABF2/AREB1* and *ABF4/ABRE2* are upregulated by dehydration and exogenous ABA (Choi et al. 2000; Kang et al. 2002; Uno et al. 2000), ultimately activating downstream genes.

Transgenic plants overexpressing *Arabidopsis ABF3* or *ABF4* were significantly more tolerant to water deficit than wild type in *Arabidopsis* (Kang et al. 2002) or in rice (Oh et al. 2005), and their seeds were hypersensitive to ABA in germination assays (Kang et al. 2002). Both *35S:ABF3* and *35S:ABF4* transgenic *Arabidopsis* exhibited reduced stomatal apertures compared to wild type *Arabidopsis* (Kang et al. 2002), indicating that drought tolerance of these transgenic plants is likely due to reduced water loss through the stomata. In plant species other than *Arabidopsis*, several AREB-like proteins were identified and used successfully to increase abiotic stress tolerance such as drought or salt stress (Hsieh et al. 2010; Jin et al. 2009; Kerr et al. 2017; Orellana et al. 2010; Wang et al. 2016). In tomato, two AREB-like proteins, *SIAREB1* and *SIAREB2*, were identified and overexpression of *SIAREB1* increased drought and salt tolerance of *Arabidopsis* or tomato (Hsieh et al. 2010; Orellana et al. 2010). Also, constitutive expression of ABFs/AREBs homologs from other plant species such as wheat *TaAREB3* (Wang et al. 2016) and cotton *GhABF2D* (Kerr et al. 2017) enhanced drought tolerance, suggesting that ABF/AREB is functionally well conserved across plant species.

Higher level of stress-related transcription factors such as *ABF/AREBs* commonly causes undesirable dwarf phenotype (Kang et al. 2002; Kerr et al. 2017). As exogenous ABA inhibits plant growth, the dwarf phenotype of *35S:ABF3* and *35S:ABF4* transgenic *Arabidopsis* plants may have been due to an ABA-hypersensitive response caused by the transgene (Kang et al. 2002), and/or the persistently lower stomatal aperture resulted in reduced carbon fixation causing dwarfing. A similar phenotype was also observed in plants constitutively expressing stress-related transcription factors such as *LeCBF1*, *AtCBF1*, and *AtCBF3*

(Gilmour et al. 2000; Hsieh et al. 2002; Knight et al. 2004; Zhang et al. 2004). However, dwarf phenotypes caused by strong *CaMV 35S* promoter were not observed in transgenic plants expressing stress-responsive genes such as *AtABF4* or *AtCBF3* under the control of the *ubiquitin1* promoter (Oh et al. 2005) or ABA-/drought-responsive *RD29A* promoter (Kasuga et al. 2004), indicating promoter selection as probably the most crucial parameter to obtain desirable outcomes without deleterious side effects. Since most water loss in plant occurs through stomatal pores, alternative way to improve drought tolerance can be targeted drought-responsive gene expression in guard cells. It was suggested that targeted gene expression under the control of a guard-cell-specific and drought-responsive promoter could be a very effective way to improve drought tolerance while minimizing side effects (Na 2005; Na and Metzger 2014). Recently, a few studies reported that guard-cell-specific targeted gene expression enhanced plant drought tolerance (Chen et al. 2016; Kelly et al. 2013; Lu et al. 2013; Lugassi et al. 2015; Na 2005). In these studies, guard-cell-specific promoter such as *KST1*, *GCI*, *KATI*, or *RAB18* promoter was used to induce stress-responsive genes in guard cells. Among them, *RAB18* promoter showed guard-cell specificity and responsiveness to exogenous ABA.

In this study, we investigated whether guard-cell-specific expression of an *Arabidopsis* ABRE-binding protein, *ABF4* (Choi et al. 2000; Uno et al. 2000), could improve drought tolerance of tobacco and tomato plants while minimizing deleterious effects on growth. We found that the expression of *ABF4* under the control of the guard-cell-specific *KST1* promoter (Plesch et al. 2001) was sufficient to increase drought tolerance in both crop species by reducing transpiration while significantly minimizing the dwarf phenotype caused by transgene expression, as compared to *Arabidopsis* plants with *35S:ABF4* (Kang et al. 2002).

## Materials and methods

### Generation of transgenic plants with *KST1* (*35S*):*ABF4-mGFP*

A 1.4 kb of a full-length cDNA for *Arabidopsis ABF4* gene was amplified by gene-specific primer pairs (F: 5'-ACGCGTCGACATGGGAACCTCACATCAATTTTC-3', R: 5'-ACAGTCATGAACCATGGTCCGGTTAA

TGTC-3'), and a PCR fragment was cloned into the Sall/Nco1 site of the pCambia 1302 binary vector to generate the *ABF4-mGFP* fusion gene. Then, a 0.3-kb *KST1* PCR fragment or the *CaMV 35S* promoter from a modified pUC18 vector (Sheen et al. 1995) was ligated into the HindIII/Sall sites to generate the *KST1(35S):ABF4-mGFP* construct. The 0.3-kb *KST1* promoter having a guard-cell-specific element (Plesch et al. 2001) was amplified from potato genomic DNA using *KST1*-specific primer pairs (F: 5'-CGCG GATCCATCTGCGTACAGTCTACCT -3', R: 5'-CATGCCATGGGTCGACTATTATATATTGCTGC TTCTTT-3'). The PCR fragment was first cloned at BamH I/Nco1 sites of modified pUC18 vector, digested with Hind III/Nco1, and the digest having *KST1* promoter was cloned into the pCambia 1302 vector. Both pCambia vectors harboring *KST1(35S):ABF4-mGFP* were transferred into *Agrobacterium tumefaciens* GV3101 by electroporation and used for transformation of tobacco and tomato by following methods described previously (Horsch et al. 1998; Park et al. 2003). The transgenic *KST1(35S):ABF4-mGFP* plants having resistance to hygromycin (30 mgL<sup>-1</sup>) were selected and then verified for single-locus T-DNA insertion by examining 3:1 ratio of antibiotic (hygromycin) resistance. Homozygous transgenic tobacco lines were obtained from transgenic tobacco lines with 3:1 ratio of antibiotic resistance and used for further analysis. As for tomato, however, T<sub>2</sub>-segregating tomato lines with 3:1 ratio of antibiotic resistance were used for phenotypic analysis. Total RNA was isolated from 3-week-old tobacco seedlings in MS medium using an RNeasy® Plant Mini Kit (Qiagen USA, Valencia, CA) or from 1-month-old tomato plants in the greenhouse. Transgene expression was examined by RT-PCR (Takara, Madison, WI) using the following primers: forward primer 5'-ATGG GAACTCACATCAATTC-3', reverse primer 5'-ACAGTCATGAACCATGGTCCGGTTAATGTC-3'. GFP and brightfield images were photographed under the PCM2000 confocal laser scanning microscope (Nikon).

#### Protein extraction and Western blot analysis

Total protein was extracted from 1-month-old leaf tissues (approximately 100 mg) of wild type or transgenic tobacco plants. To extract protein, leaf tissues were ground using a mortar and pestle after adding 500 µL of SDS extraction buffer (0.125-M tris-HCl, 4% SDS,

20% glycerol, 2% 2-mercaptoethanol, pH 6.8). The slurry was transferred into a 2-mL Eppendorf tube and centrifuged for 3 min at 10,000 rpm at 4 °C. The supernatant was transferred to a new tube and mixed with 1.5 mL of 100% acetone. After 30-min incubation at 4 °C, the samples were centrifuged for 3 min at 10,000 rpm at 4 °C and the pellet was dissolved in 2X SDS protein loading buffer. Then, an equal volume of each sample was separated on 8% SDS-PAGE gel. After electrophoresis, the gel was stained by Coomassie blue staining buffer, and protein levels were determined based on intensity. Protein was transferred to poly (vinylidene difluoride) membrane (Bio-Rad) by standard techniques (Sambrook et al. 1989). The membrane was blocked in 1X TBS (10-mM Tris HCl, pH 8.0/150-mM NaCl) containing 1% (wt/vol) non-fat dry milk at room temperature for 1 h, incubated with anti-GFP rabbit polyclonal antibody (Molecular Probes, Eugene, OR, USA), and developed using peroxidase-conjugated secondary antibody (dilution ratio of 1:5000) by enhanced chemiluminescence (Amersham Pharmacia).

#### Measurement of water potential and relative water contents

For tobacco, two *KST1:ABF4-mGFP* transgenic (NK3 and NK27) and wild-type plants were grown in 2-L pots in the greenhouse for 7 weeks. After that, three plants from each of the three genotypes were used to measure the water potential using a WP4-T dewpoint potentiometer (Decagon, Pullman, WA, USA). For tomato, the water potential of 2-month-old tomato plants in the greenhouse was measured using a Scholander-type pressure chamber (the Model 1000 pressure chamber, PMS, Albany, OR, USA). Three to five plants from T<sub>2</sub> heterozygous *KST1(35S):ABF4* and wild-type plants were used for measurement of water potential between 12:00 pm to 2:00 pm (non-stressed conditions) or 4:00 pm to 6:00 pm (water-stressed conditions). At the same time, leaf tissues of similar size were collected from each plant to measure the relative water content (RWC).

To determine RWC, leaf tissues were used to measure fresh weight (FW) and turgid weight (TW). For FW, leaf tissues were weighed immediately after harvest, and for TW, the tissue was fully hydrated by placing it in a 15-mL polyethylene cup with 3–5 mL of distilled water and placed in a refrigerator at 4 °C for 1 day, and the weight was re-measured. The tissue was

then dried in an oven at 60 °C for 1 day and the dry weight (DW) was determined. RWC was calculated using the following equation:  $RWC = (FW - DW) / (TW - DW)$ .

### Stress tolerance

Two *KST1:ABF4-mGFP* transgenic and wild-type tobacco seeds were sown in 10-cm diameter pots filled with soilless media (Metro-Mix 360, The Scotts-Sierra Co., Marysville, OH, USA). Pots were placed in the greenhouse with 16-h light conditions with regular irrigation for the first month. To apply a water deficit, water was withheld for 20 days. After 20-day water deprivation, pots with plants were re-watered and pictures were taken after 5-day recovery. Early wilting was observed from 2-month-old tobacco and 1-month-old tomato plants by withholding water for 1 day.

To examine how transgenic tomato plants respond to periodical water deficit, 10 seeds of each wild type (WT), *KST1:ABF4-mGFP* (LK34) or *35S:ABF4-mGFP* (LS41) T<sub>2</sub>-segregating tomato plants, were sown in 2-L pots and grown for a month in the greenhouse. In the following month, these plants were treated with a 3- to 5-day deprivation of water each week until they exhibited severe wilting symptom. Plants were photographed after 1-month recovery with regular watering from severe water deficit.

### Measurement of plant water usage

Two-week-old transgenic and wild-type tobacco or tomato seedlings in MS (Sigma) media were individually transferred into 2-L pots filled with soilless media and grown in the greenhouse for 2 months. For tobacco, 6 plants from transgenic or wild-type plants were harvested prior to recording daily water loss as a reference for leaf area, height, leaf number, and dry and fresh weight. Daily evapotranspiration was determined by lysimetry. Pots containing plants were weighed using a top loading balance twice a day at 11:00 am and 12:30 pm for 10 days for tobacco and for 23 days for tomato. The plants were irrigated each day at 11:30 am. Daily evapotranspiration was determined by calculating the difference in the weights of the pot at 12:30 pm and the weight at 11:00 am the following day. Transpiration was deduced by subtracting the average amount of evaporation from three 2-L pots filled with soilless media without plants. Transpiration per leaf unit area

was determined by dividing water loss per each 2 h by the total leaf area.

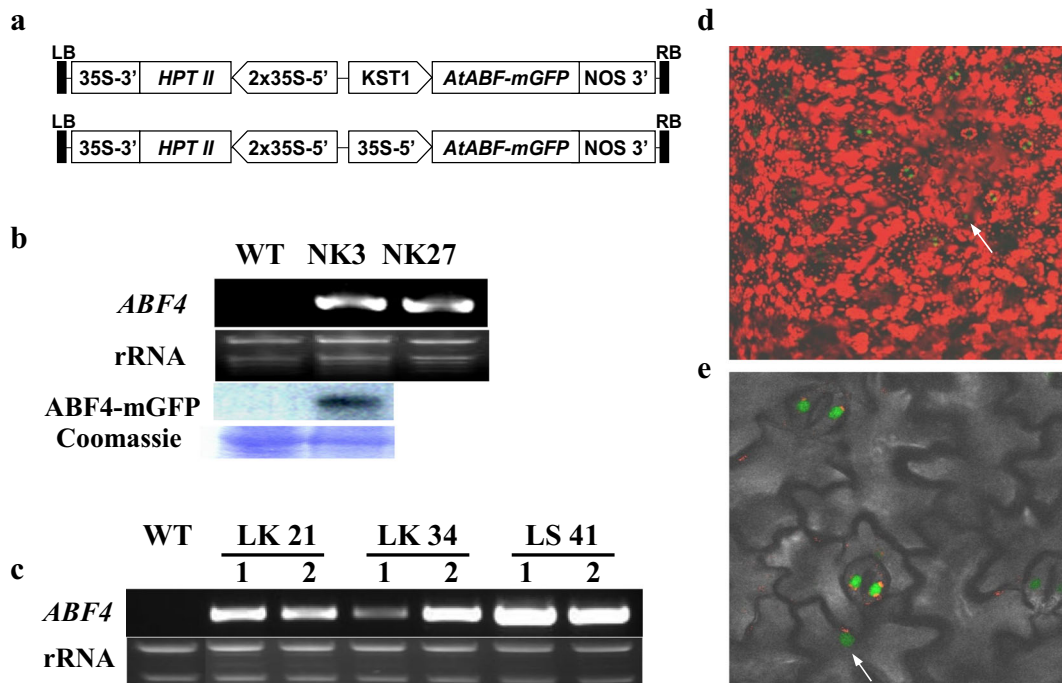
## Results and discussion

### Generation of transgenic tobacco and tomato plants with *Arabidopsis ABF4*

To study effects of the expression of the *Arabidopsis ABF4* gene in guard cells, tobacco and tomato were transformed with pCambia-1302 binary vectors carrying an *ABF4-mGFP* fusion gene construct under the control of the guard-cell-specific 0.3-kb *KST1* promoter (Muller-Rober et al. 1995; Plesch et al. 2001) or strong *35S CaMV* promoter (Fig. 1a). For tobacco, 11 *KST1:ABF4-mGFP* transgenic lines were obtained from hygromycin selection and two T<sub>3</sub> transgenic tobacco lines (designated NK3 and NK27) were used for further study. For tomato, two segregating T<sub>2</sub> *KST1:ABF4-mGFP* lines (LK21 and LK34) and one segregating T<sub>2</sub> *35S:ABF4-mGFP* line (LS41) were used for further analysis. The expression of transgene *ABF4* was examined by RT-PCR and only detected in the transgenic tobacco or tomato plants (Fig. 1b, c). The expression of *ABF4-mGFP* was analyzed by Western blot analysis, and the expected 75-kD fusion protein was detected in transgenic tobacco plant using polyclonal GFP antibody (Fig. 1b).

### *ABF4* accumulates strongly in nuclei of guard cells of transgenic tobacco plants

Transcription factor *ABF4* activates dehydration-responsive genes such as *RD29B* upon binding on ABRE elements in their promoters, as was shown previously in in vitro or transient expression assays (Kang et al. 2002; Uno et al. 2000). To visualize the cellular localization of *ABF4* in plants, *ABF4* was fused to the *mGFP* reporter gene as shown in Fig. 1a. Heterologous *ABF4-mGFP* fusion protein accumulated in nuclei of guard cells in transgenic tobacco plants (Fig. 1d, e). The localization in guard cells is consistent with the previous demonstration that the *KST1* promoter led to strong expression of *GUS* reporter gene in guard cells (Plesch et al. 2001). However, the *ABF4-mGFP* protein was also detected in the nuclei of epidermal cells, although it was weaker than in guard cells as indicated by an arrow (Fig. 1d, e). Nonetheless, the localization in nuclei



**Fig. 1** Analysis of transgene expression in tobacco and tomato plants. **a** Construct used for plant transformation. *Arabidopsis* ABF4 gene was placed under the control of constitutive *CaMV* 35S or potato *KST1* guard-cell-specific promoter. **b** RT-PCR analysis of *ABF4* expression in wild type (WT) and two *KST1:ABF4-mGFP* homozygous transgenic tobacco lines (NK3, NK27). The expression of ABF4-mGFP fusion protein in NK3 transgenic plants was determined by Western blot with anti-GFP antiserum.

**c** RT-PCR analysis of *ABF4* expression in WT, *KST1:ABF4-mGFP* (LK21 and LK34) and *35S:ABF4-mGFP* T<sub>2</sub> transgenic tomato (LS41). Numbers (1 and 2) below LK21, LK34, and LS41 indicate two different transgenic lines used for RT-PCR analysis. **d** Localization of ABF4-mGFP fusion proteins in NK3 tobacco plant was observed under the PCM2000 confocal laser scanning microscope (Nikon). **e** Magnified and superimposed image of (**d**)

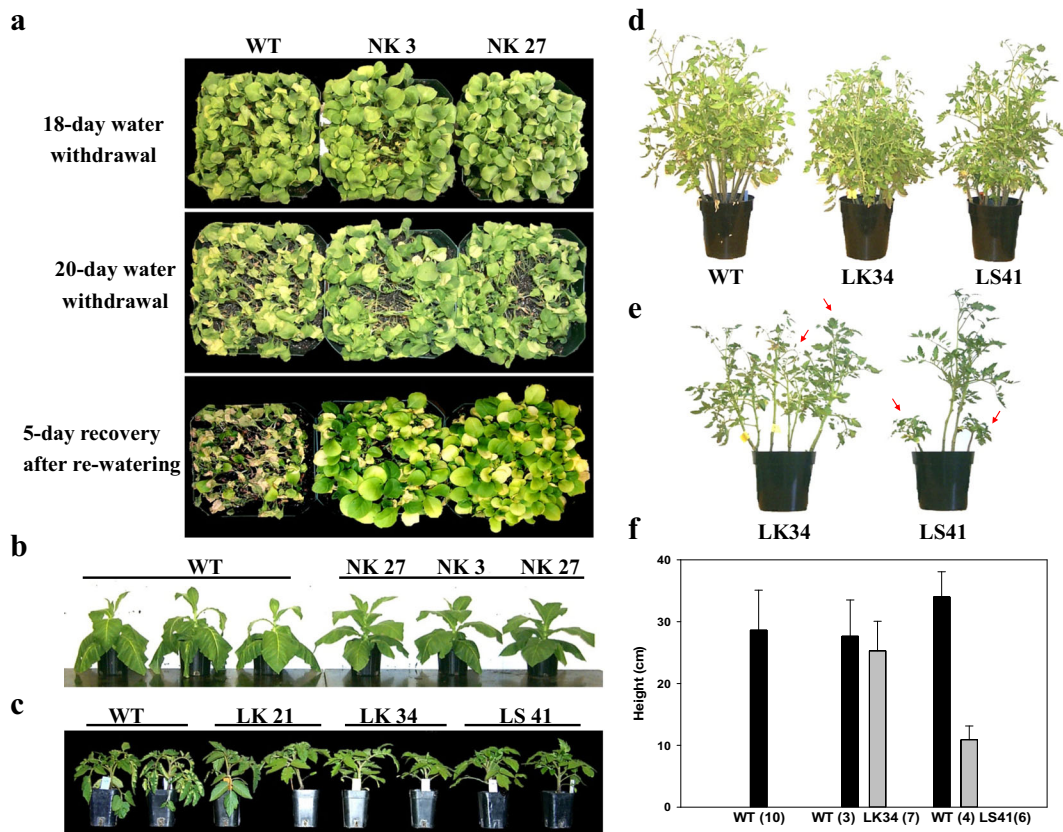
is consistent with result from ABF4 transactivation assays in that ABF4 activates ABA-responsive genes upon binding to ABRE elements (Kang et al. 2002; Uno et al. 2000).

Guard-cell-specific *ABF4* expression improves drought tolerance

To examine whether *ABF4* expression in guard cells improves drought tolerance as shown in *Arabidopsis* plants constitutively expressing *ABF3* or *ABF4* (Kang et al. 2002; Kim et al. 2004), *KST1:ABF4-mGFP* transgenic and wild-type plants were grown in pots in a greenhouse for 1 month with regular irrigation. For tobacco, transgenic plants, NK3 and NK27, exhibited minor wilting symptoms when water was withheld for 18 days, whereas wild-type plants were severely wilted. After 20 days, however, all lines had severely wilted (Fig. 2a). To test the ability to recover from the 20-day water deficit, the plants were irrigated and pictures taken 5 days later.

Both transgenic lines, NK3 and NK27, recovered within 5 days after irrigation, whereas most wild-type plants were dead or did not fully recover (Fig. 2a).

We also examined whether ABF4 transgenic tobacco and tomato plants exhibited drought tolerance at an older stage of development during a typical sunny day when daily irrigation could not keep up with evapotranspiration. In 2-month-old wild-type tobacco plants, severe wilting was observed earlier than in *KST1:ABF4-mGFP* transgenic plants during the day (Fig. 2b). Also, as with tobacco, a similar result was observed in transgenic tomato plants. When wild-type tomato plants exhibited leaf bending as an early wilting symptom, *KST1:ABF4-mGFP* and *35S:ABF4-mGFP* transgenic plants were not affected (Fig. 2c), indicating that the transgenic plants are more resistant to water deficit. These results are consistent with previous study, in which both *Arabidopsis* with *35S:ABF4* or *35S:ABF3* and rice plants with *ubi1:ABF3* exhibited tolerance to water deficit (Kang et al. 2002; Oh et al. 2005).



**Fig. 2** *KST1:ABF4-mGFP* transgenic plants were more tolerant to water deficit than wild-type plants. **a** Water deficit and recovery of WT and transgenic tobacco plants, NK3 and NK27 ( $n = \pm 100$ ). **b** Two-month-old WT tobacco and **c** one-month-old WT tomato exhibited wilting symptom earlier than *KST1/35S:ABF4-mGFP* transgenic plants. **d** Pots containing 10 1-month-old plants of each WT tomato,  $T_2$ -segregating *KST1:ABF4-mGFP* (LK34), or  $T_2$ -segregating *35S:ABF4-mGFP* (LS41) were periodically water-

stressed by stopping irrigation for 1 month and followed by recovery for a month with regular watering. **e** Two transgenic and WT tomato plants were photographed to show the difference in growth between transgenic and WT from **(d)**, and the red arrows indicate transgenic plants, LK34 and LS41. **f** The average height of transgenic or WT tomato plants from each pot from **d** was shown in graph. Number in parenthesis indicates number of WT or transgenic plants among 10 plants

Also, these results suggest that the expression of *ABF4* under the control of the guard-cell-specific promoter *KST1* improved drought tolerance in both species. However, the extent to which the low level of expression observed in epidermal cells of *KST1:ABF4-mGFP* plants contributes to the increase in drought tolerance is unknown (Fig. 1e, f). Recently, several studies reported that targeted gene expression in guard cells reduced water loss. Kelly et al. (2013) reported that *hexokinase (HXK)* expression in guard cell using *KST1* promoter decreased water loss by transpiration in tomato plants. Also, *phospholipase Da1 (PLD $\alpha$ 1)* expression under the control of guard-cell-specific *KAT1* promoter reduced water loss while increasing seed production of canola, *Brassica napus*, under drought (Lu et al. 2013).

Another study reported that *Arabidopsis* plants expressing a tobacco vacuolar invertase inhibitor, *Nt-inhh*, using an ABA-inducible and guard-cell-specific *AtRab18* promoter, improved drought tolerance (Chen et al. 2016).

#### *KST1:ABF4-mGFP* transgenic plants exhibit mild pleiotropic growth effects

Although a stunted phenotype was not observed in early stages of development in either the transgenic tobacco or tomato plants (Fig. 2b, c), *KST1:ABF4-mGFP* tobacco (Tables 1 and 2) and *KST1(35S):ABF4-mGFP* tomato plants (Fig. 2b, c, d) exhibited mild growth reduction at later stages of development. The growth of the

**Table 1** Summary of growth parameters in 3-month-old *KST1:ABF4* transgenic tobacco plants

Lines	Leaf no.	Leaf area (cm <sup>2</sup> )	Fresh weight (g)	Dry weight (g)	Height (cm)
WT	14.3 ± 0.5	3237 ± 158a	166 ± 9.9a	17.4 ± 1.1a	41 ± 1.7a
NK3	14.2 ± 0.4	2977 ± 114b	151 ± 7.5b	14.7 ± 0.7b	33 ± 1.4b
NK27	14.2 ± 0.6	3001 ± 144b	150 ± 8.0b	14.5 ± 1.0c	34 ± 2.9b

The means ± standard deviations are shown,  $n = 12$ . Different letters within a column denote significant differences within the three genotypes ( $P < 0.05$ ) for Tukey pairwise multiple comparison

transgenic tobacco plants was reduced in leaf area by 8%, fresh weight by 9%, dry weight by 16%, and height by 18% compared to wild-type plants over a 3-month experimental period (Tables 1 and 2). Interestingly, these growth reductions were mainly attributed to those obtained during the last 10-day experimental period (Tables 1 and 2), suggesting that the stunting phenotype may be initiated depending on developmental progression after a certain amount of time and/or that the persistent presence of transgene may increase a sensitivity of the transgenic plants to water deficit.

In a previous study, *Arabidopsis* expressing *35S:ABF4* showed stunting, the severity of which correlated with the expression level of the *ABF4* gene (Kang et al. 2002). Therefore, the mild stunting observed in *KST1:ABF4-mGFP* transgenic tobacco and tomato plants may be due to a relatively low expression level of the transgene *ABF4*. Clearly, transcript levels of *ABF4* in *35S:ABF4-mGFP* tomato plants were higher than the levels in the *KST1:ABF4-mGFP* lines, LK21 and LK34 (Fig. 1c), and the *35S:ABF4-mGFP* lines exhibited a slightly more severe stunting than the *KST1:ABF4-mGFP* tomato plants, especially with water deficit (Fig. 2e), consistent with the observation that this phenotype in *Arabidopsis* was correlated with *ABF4* expression driven by the *35S* constitutive promoter

(Kang et al. 2002). Another possible explanation is that the *ABF4* transgene in either tobacco or tomato may require modification to exert its maximum effect to induce the expression of downstream-responsive genes. In *Arabidopsis*, *ABF2* and *ABF4* were highly activated by phosphorylation in an ABA-dependent manner (Choi et al. 2005; Furihata et al. 2006), suggesting that *ABF4* transgenic tobacco or tomato plants may yet develop a more severe stunted phenotype with an increase in the endogenous ABA under water deficit.

Therefore, we had examined whether long-term water deficit would result in a stunting phenotype in the transgenic plants. For the test, water deficit was carried out with both *35S:ABF4-mGFP* and *KST1:ABF4-mGFP* tomato plants by repeating a 3- to 5-day deprivation of water each week for a month. The *KST1:ABF4-mGFP* transgenic and wild-type tomato plants did not show any significant change in growth in the experiment. However, *35S:ABF4-mGFP* tomato plants exhibited a very severe stunted phenotype and did not recover from the water deficit in the month following with regular irrigation (Fig. 2d, e; LS41). *KST1:ABF4-mGFP* tomato plants showed insignificantly lower growth during the prolonged water deficit compared to wild type, while the *35S:ABF4-mGFP* plants were severely reduced in height and biomass (Fig. 2f). This suggests that guard-cell-

**Table 2** Summary of plant growth during a 10-day period. Water use efficiency (WUE) was calculated by dividing the increase of dry weight by total water loss during the 10-day experiment period

Lines	Leaf area Increase (cm <sup>2</sup> )	Fresh weight increase (g)			Dry weight increase (g)			Total water loss (kg)	WUE
		Leaf	Stem	Total	Leaf	Stem	Total		
WT	1703 ± 158a	56 ± 6.4a	43 ± 3.8a	99 ± 9.9a	7.9 ± 0.8a	3.9 ± 0.3a	11.8 ± 1.1a	2.3 ± 0.12	5.1
NK3	1515 ± 114b	51 ± 5.0a	33 ± 2.7b	84 ± 7.5b	6.4 ± 0.5b	3.0 ± 0.3b	9.4 ± 0.7b	1.8 ± 0.10	5.2
NK27	1550 ± 144b	50 ± 4.8b	35 ± 3.8b	85 ± 8.0b	6.3 ± 0.7b	3.1 ± 0.3b	9.4 ± 1.0b	1.8 ± 0.09	5.2

WUE: dry weight gain (g)/water loss (kg)

The means ± standard deviations are shown,  $n = 12$ . Different letters within a column denote significant differences within the three genotypes ( $P < 0.05$ ) for Tukey pairwise multiple comparison

specific expression of *ABF4* does not interfere with normal growth and probably that the mild stunting phenotype of *KST1:ABF4-mGFP* plants is attributable to leaky *ABF4* expression in other cell types (Fig. 1d, e). Similarly, growth inhibition was compared by expressing *HXK* gene under the control of *35S* or guard-cell-specific *KST1* promoter (Kelly et al. 2013), in which *35S:HXK* plants did show dwarfism while *KST1:HXK* plant grew normal like wild type, indicating that guard-cell-specific stress-responsive gene expression is useful tool to minimize dwarfism.

#### *ABF4* transgenic plants conserve water by reducing transpiration

Since transgenic tobacco and tomato plants expressing *ABF4-mGFP* in guard cells exhibited drought tolerance, we examined whether the drought tolerance of these transgenic plants was due to altered stomatal activity. Daily water loss was measured by weighing pots with 2-month-old *KST1(35S):ABF4-mGFP* transgenic or wild-type plants. Daily water use patterns of transgenic tobacco (Fig. 3a) and tomato (Fig. 3b) plants were significantly lower than those of their wild-type counterparts. In addition, transgenic plants transpired less water per 100-cm<sup>2</sup> unit leaf area than wild-type plants under normal conditions (Fig. 3c, d), and this result was coincident with the daily water use pattern of transgenic plants, indicating that lower daily water use of transgenic plants is likely due to reduced transpiration. The expression of *ABF3* or *ABF4* in *Arabidopsis* (Kang et al. 2002) and *ABF3* in rice (Oh et al. 2005) conferred drought tolerance, and *Arabidopsis* with *35S:ABF3* or *35S:ABF4* showed reduced stomatal aperture (Kang et al. 2002), suggesting that both tomato and tobacco *KST1:ABF4-mGFP* plants may reduce transpiration via reduction in stomatal aperture.

Water use efficiency (WUE) of *KST1:ABF4-mGFP* tobacco plants was examined by dividing the gain of dry weight by the amount of water used during 10-day experimental period. Although WUE of transgenic tobacco plants was slightly higher than wild type, the difference was not significant (Table 2). It is unclear whether an extended experimental period can result in more difference. On the other hand, it can be speculated that *ABF4* expression in guard cells does not affect WUE, but it may cause reduced stomatal aperture, resulting in reduced water loss. Consequently, the reduced stomatal aperture might lead lower CO<sub>2</sub>

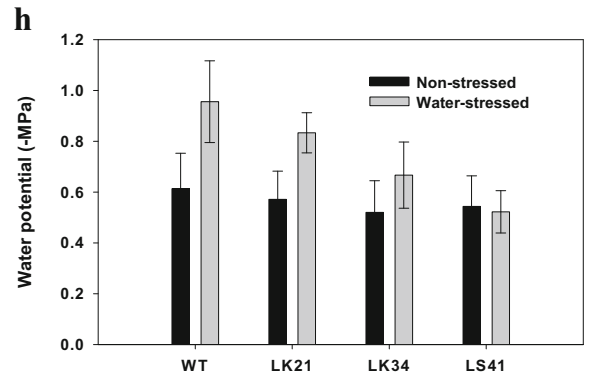
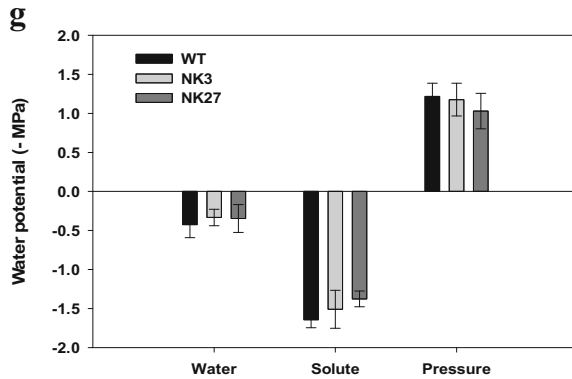
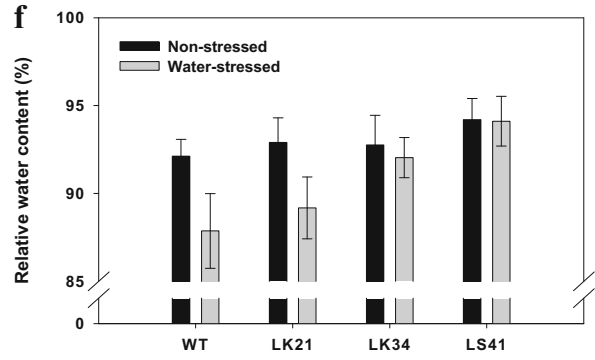
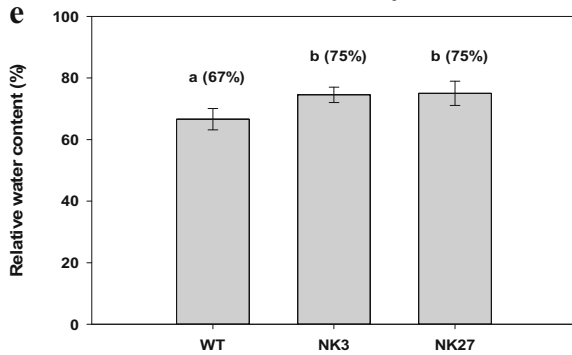
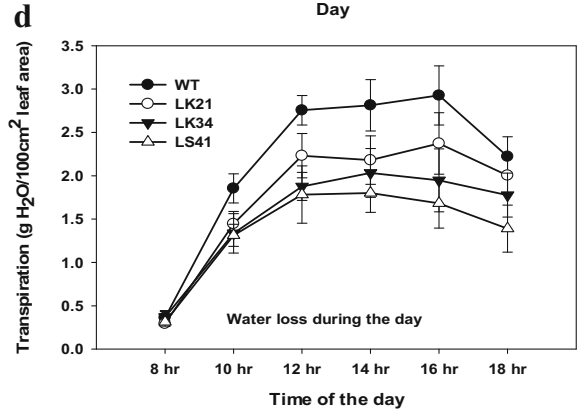
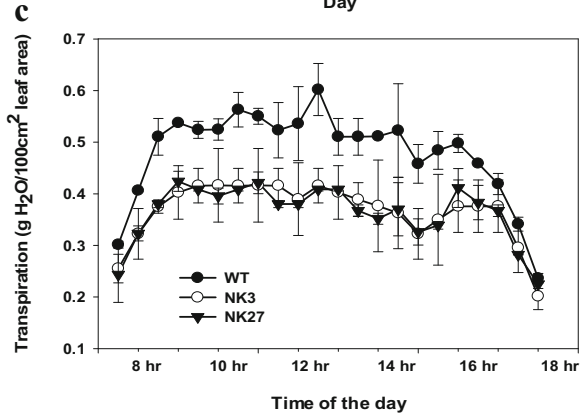
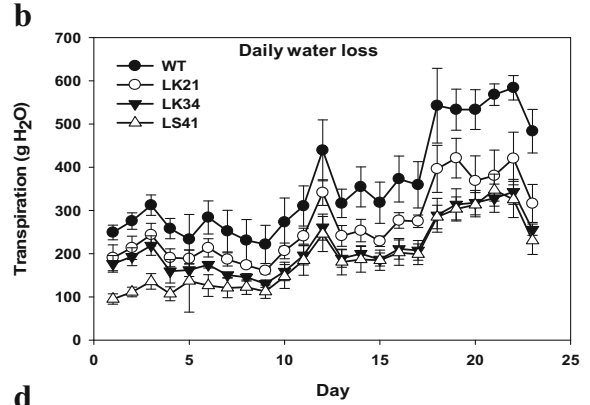
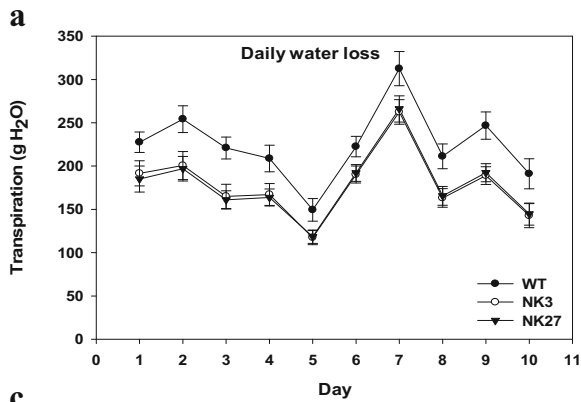
**Fig. 3** *KST1(35S):ABF4-mGFP* transgenic plants conserve significantly more water than WT. Daily water loss of **a** NK3 and NK27 tobacco plants with *KST1:ABF4-mGFP* or **b** transgenic tomato plants LK21 and LK34 with *KST1:ABF4-mGFP* or LS41 with *35S:ABF4-mGFP* was much less than that of WT ( $n = 12$  for tobacco,  $n = 4$  to 5 for tomato). Time-course of transpiration of **c** 3-month-old transgenic and WT tobacco plants or **d** 3-month-old tomato plants during the day ( $n = 4$  for tobacco and  $n = 4$  to 5 for tomato). Relative water content of **e** tobacco or **f** tomato plants. Water potential of **g** tobacco and **h** tomato plants. In **f** and **g** graphs, relative water content (RWC) and water potentials were measured at non-stressed conditions between 12:00 pm and 2:00 pm and water-stressed conditions between 4:00 pm and 6:00 pm. Vertical bars denote the mean  $\pm$  SD ( $n \geq 3$  plants in triplicates for water potential,  $n = 6$  plants in duplicate for relative water content)

assimilation due to reduced CO<sub>2</sub> uptake, and in turn results in a mildly stunted phenotype of *KST1:ABF4-mGFP* plants without changing WUE.

Relative water content (RWC) and water potential of transgenic tobacco plants expressing *KST1:ABF4-mGFP* were measured only under well-watered conditions. RWC was significantly higher in transgenic tobacco than wild type (Fig. 3e), which was not consistent with *KST1:ABF4-mGFP* tomato plants that did not exhibit any difference in RWC from wild type without water deficit (Fig. 3f). However, water potential in transgenic tobacco versus wild type was not different (Fig. 3f), consistent with water potential of transgenic tomato plants under non-stressed conditions between 12:00 pm and 2:00 pm.

RWC of *KST1:ABF4-mGFP* transgenic tomato and wild type was similar under non-stress conditions during 12:00 to 2:00 pm in the early afternoon, but it was significantly higher in transgenic tomato than wild type under water-stressed conditions between 4:00 to 6:00 pm in the late afternoon (Fig. 3g) when wild type exhibited minor wilting. Consistent with RWC, water potential of both transgenic and wild-type tomatoes was similar under non-stressed conditions, but it was significantly higher in the transgenic tomato plants at the end of the day (water-stressed conditions during 4:00 pm to 6:00 pm) (Fig. 3h). These results make sense in that high transpiration of wild type can result in the depletion of water earlier than in *KST1:ABF4-mGFP* transgenic plants. As a result, transgenic plants can maintain higher water content as well as higher water potential longer than wild type, which can be critical for plant survival during periodic water deficit in field performance.





In summary, several studies have demonstrated that the guard-cell targeted manipulation of genes related to drought-adaptive mechanisms can be a useful tool to improve plant drought tolerance. Consistently with previous observations, we found that the expression of *ABF4* in guard cells reduces transpiration and significantly improves drought tolerance of transgenic tobacco and tomato plants while minimizing undesirable side effects such as stunted growth.

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