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ORIGINAL PAPER

Arabidopsis TEMPRANILLO1 transcription factor AtTEM1 negatively regulates drought tolerance

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Abstract Arabidopsis TEMPRANILLO 1 (*AtTEM1*) is a member of the RAV transcription factor subfamily and has plant-specific AP2 and B3 domains. This study demonstrated that *AtTEM1* is localized in nuclei. Expression of *AtTEM1* was discovered to be high in the flower, stem, rosette, and cauline leaf, but low in the silique and root tissue, and was strongly induced by drought and mannitol treatments. Compared with wild-type plants, the 35S::*AtTEM1* plants bolted later and produced more rosette leaves before flowering, whereas, the *AtTEM1* mutant plants (*tem1*) bolted earlier but produced an almost equal number of rosette leaves before flowering. Drought tolerance was significantly reduced in the 35S::*AtTEM1* transgenic *Arabidopsis* plants, but was enhanced in the *tem1* plants. Under drought and mannitol stresses, the 35S::*AtTEM1* transgenic *Arabidopsis* exhibited increased relative electrical conductivity (REC), increased malondialdehyde (MDA) content, and decreased proline content compared with the wild type, whereas lower REC and MDA content and higher proline content were found in the *tem1* plants. These results suggest that AtTEM1 in *Arabidopsis* functions as a negative regulator in response to drought and mannitol stresses. Therefore, AtTEM1 transcription factor is proposed to be a versatile regulator reacting negatively to both flowering time and drought stress.

Keywords *Arabidopsis* · *AtTEM1* · TEMPRANILLO1 transcription factor · Drought tolerance · Mannitol tolerance

Introduction

Plants cannot escape unfavourable environmental conditions within their typical life cycle (Kang et al. 2011) and are under stresses when their natural environment deteriorates, such as when there is a drought (Achard et al. 2008). Environmental stresses can severely damage the cellular structure of plants and impair their physiological function (Larcher 2003). To survive adverse environmental conditions, plants develop stress tolerance mechanisms to maintain growth and reproduction (Sato et al. 2014). Many stress signals triggered in plants can cause biochemical and physiological changes, resulting from stress-induced modulation of gene expression (Achard et al. 2008).

The B3 transcription factor family is a large group of plant-specific transcription factors involved in controlling the primary and secondary metabolism, growth and developmental programmes, and responses to environmental stimuli (Licausi et al. 2013). In *Arabidopsis*, these transcription factors are encoded by 118 genes that can be classified into four major subfamilies: LAV *(LEAFY COTYLEDON2 [LEC2]–ABSCISIC ACID INSENSITIVE3 [ABI3]–VAL)*, ARF (AUXIN RESPONSE FACTOR), RAV (RELATED TO ABI3 and VP1) and REM (REPRODUCTIVE MERISTEM) (Swaminathan et al. 2008).

Some RAV subfamily transcription factors have been functionally characterized, including RAV1, RAV1-like,

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RAV2/TEMPRANILLO2 (TEM2) and TEM1. Overexpression of *RAV1* can reduce the number of lateral roots and rosette leaves, indicating that *RAV1* acts as a negative regulator during plant development (Hu et al. 2004). *TEM* transcription factors are involved in controlling the flowering time for photoperiodic induction. *TEM* genes have a pivotal role in the direct repression of *FLOWERING LOCUS T* (*FT*), which links photoperiod and gibberellin pathways to control flowering in *Arabidopsis* (Osnato et al. 2012). Overexpression of *TEM1* or *TEM2*/*RAV2* in *Arabidopsis* was demonstrated to delay its flowering time for several weeks by directly repressing *FT* expression (Castillejo and Pelaz 2008). Brassinosteroids (BR) was revealed through cDNA microarray analysis to possibly regulate RAV1 through a BRI1-independent signal pathway in *Arabidopsis. RAV1* may function as a negative regulatory component of growth and development (Hu et al. 2004). Furthermore, transgenic cotton coexpressing the *Arabidopsis* B3-domain transcription factor *AtRAV2* and basic leucine zipper (bZIP) *AtABI5* is highly resistant to osmotic and drought stress (Mittal et al. 2014). Overexpression of pepper *RAV1* (*CARAV1*) in *Arabidopsis* can enhance its ABA sensitivity and its resistance to bacterial pathogens, salt and drought stress (Sohn et al. 2006). *AtTEM1* is annotated as an AP2 and B3 domain-containing transcription factor. Down-regulation of *AtTEM1* can result in early flowering (Castillejo and Pelaz 2008). The present study analyzed the *Arabidopsis AtTEM1* spatial mRNA expression pattern and subcellular localization, and its effect on plant drought response. The results demonstrated that *AtTEM1* localizes to the cell nucleus and is expressed at low levels in the silique and root tissue, acts as a negative regulator of flowering time and drought derived stress response.

Materials and methods

Plant material and transformation

Arabidopsis thaliana ecotype Columbia and its transgenic plants were grown at 22 °C, with a day length of 12 h and a light intensity of 70 micromoles of photons per square meter per second, in plastic bowls (φ = 9 cm) containing a steam-sterilized soil mixture (peat moss, perlite, loam soil, and vermiculite: 5:3:2:1, v/v/v/v).

The T-DNA insertion mutants *tem1-1* and *tem1-2* were obtained from the *Arabidopsis* Biological Resource Center and were identified as homozygous using the polymerase chain reaction (PCR) with the primers SALK-F: (5′-AGA CTTGACCCTACTCCTCTGA-3′) and SALK-R: (5′-GTC GTCGTTGTCGGCTTT-3′).

To construct plasmids for generating *AtTEM1*-overexpressing *Arabidopsis* plants, the DNA fragment containing the *AtTEM1* coding region was cloned into the polylinker sites of the plant expression vector pCAMBIA1301m under the control of the CaMV 35S promoter by using the primers TEM1-35-F: (5′-CCCGGGACC CATTTCTTCTTCTTT-3′) and TEM1-35-R: (5′-CAG CTGGGAGGAATTAGATTATTAGAAC-3′). The plasmid pCAMBIA1301m: *AtTEM1* was introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into *Arabidopsis* using the floral dip method (Clough and Bent 1998). Transformants were selected by planting the seeds of the transformed plants in Murashige and Skoog (MS) medium containing 50 mg L^{-1} hygromycin B (Murashige and Skoog 1962). After 2 weeks, the hygromycin B-resistant seedlings were transplanted into soil.

Spatial expression pattern of *AtTEM1* in *Arabidopsis thaliana*

Total RNA was extracted from roots, stems, rosette leaves, cauline leaves, inflorescences, and pods throughout the growth period. The Applied Biosystems Veriti™ 96-well thermal cycler was used for performing reverse transcription PCR (RT-PCR).

Transient expression of *AtTEM1* in onion epidermal cells

For the GFPS65T:*AtTEM1* construction, the *AtTEM1* coding region was amplified through PCR by using the primers TEM1-G-F (5′-GCTCTAGAATGGAATACAGCTGTG TAGACG-3′) and TEM1-G-R (5′-GGATCCCGTCACAAG ATGTTGATAATCGCC-3′) to introduce *Xba*I at the 5′ end and *BamH*I at the 3′ end. The resulting fragment was digested with *Xba*I and *BamH*I and ligated in frame at the 3′ end of a green fluorescent protein (GFP) linearized with *Xba*I and *BamH*I. The *Agrobacterium tumefaciens*-mediated transfection method was used for the transient expression of GFPS65T:*AtTEM1* in the epidermal cells of the onion (*Allium cepa* L.) (Eady et al. 2000; Sun et al. 2007). The subcellular localization of the GFP fusion construct in the onion epidermal cells was monitored using an upright fluorescence microscope (Olympus BX51 Clone).

Treatment for growth development

Arabidopsis thaliana ecotype Columbia and the transgenic plants were germinated on 1/2 MS agar for 1 week and then transplanted into soil at 22 °C under long day (LD) conditions (16 h) under a normal watering regime.

Drought and mannitol stress treatments

For the drought tolerance treatment, *Arabidopsis* seeds were germinated on 1/2 MS agar for 1 week, after which the seedlings were transplanted into soil and grown under a normal watering regime for 3 weeks. Watering was then withholded for 14 days. When wild-type (WT) plants exhibited the lethal effects of dehydration, watering was resumed, and the plants were allowed to grow for a further 7 dYS. The survival rate was scored. All experiments were repeated at least three times.

To estimate mannitol stress tolerance, *Arabidopsis* seeds were placed on 1/2 MS agar plates containing 400 mM mannitol for the osmosis germination treatment. For seedling mannitol stress, WT and *AtTEM1*-transgenic 4-week-old *Arabidopsis* seedlings were planted in a soilless environment containing 400 mM mannitol and cultured under light for 12 h at 22 °C.

Measurements of REC, MDA and proline content

For the measurement of relative electrical conductivity (REC), malondialdehyde (MDA) and proline content, four-week-old seedlings were used for drought treatment, and the rosette leaves of the transgenic and WT plants during the treatment stage were harvested for the assays. The leaf REC was measured using the method described by Yu et al. (2006). MDA content was measured according to the method of Kuk et al. (2003). Free proline content was measured in acidic extracts and quantified spectrophotometrically using the acid–ninhydrin reagent with proline as a standard (Bates et al. 1973). The treatment was repeated for three times and each sample was assayed with three replicates, the standard deviations were calculated (Cheng et al. 2012).

Real-time PCR analysis

In the real-time PCR analysis treatment, *Arabidopsis* seeds were germinated on 1/2 MS agar for 1 week. The seedlings were then transplanted into soil and grown under a normal watering regime for 3 weeks. Watering was then withholded for 24 h and the total RNA was extracted from rosette leaves.

The expression of *AtTEM1* and drought-responsive genes was analyzed by real-time PCR using the following primers: FT-F (5′-CAACCCTCACCTCCGAGAATAT-3′) and FT-R (5′-TGCCAAAGGTTGTTCCAGTTGT-3′); RD22-F (5′-TAGGAGTCGGTAAAGGCGGT-3′) and RD22-R (5′-CATCGGTGCGTTCTTCTTAGC-3′); P5CS-F (5′-CCAGCTGAGCCCAACAGTGACC-3′) and P5CS-R (5′-CAGTCGGGCAGCCAGGCTATCATTATC-3′); PDH-F (5′-TCACAACCACTGAGCTAAAGTGAGA-3′) and PDH-R (5′-CGATGACGCTGTATCTTGTGATG-3′); ACTIN-F (5′-GATTTGGCATCACACTTTCTACAA TG-3′) and ACTIN-R (5′-GTTCCACCACTGAGCACA ATG-3′); AtATM3-F (5′-TGCTCGGACATTTTTGAA ATC-3′) and AtATM3-R (5′-GTCCATAGCTGCGCATAT CTC-3′). The total RNA from WT and transgenic plants was heated to 65 °C for 7 min and subjected to a reverse transcription reaction using RT-AMV transcriptase (Roche) with oligo(dT) for 1 h at 42 °C. PCR was performed using ExTaq DNA polymerase (TaKaRa, Shiga, Japan).

SYBR Green I-based real-time PCR and a melting curve analysis assay were employed using the CFX96 Touch Real-Time Q-PCR system. The 10-μL PCR mixture included 1 μL of RT product, 2× SYBR® Premix DimerEraser, and 1 μL of primer (SYBR® Premix DimerEraser™-Perfect Real Time TaKaRa). The reactions were performed in a 96-well optical plate and entailed an initial step at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 20 s. After the PCR reaction, a melting curve analysis was increased. Numerical analysis was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Data on the threshold cycle (Ct) were determined using default threshold settings and is defined as the fractional cycle number at which fluorescence reaches at fixed threshold (Livak and Schmittgen 2001).

Statistical analysis

Data were presented as mean ± standard deviation from three independent biological replicates for all assays, the statistically significant differences between the transgenic and WT plants in figures were determined with Student's *t* test using SPSS 19.0 software (IBM Corporation, Chicago, IL, USA), values of $P < 0.05$ were considered statistically significant.

Results

Spatial expression of AtTEM1 in *Arabidopsis thaliana*

Semi-quantitive RT-PCR analysis was performed to determine the spatial expression pattern of *AtTEM1*. As presented in Fig. 1, *AtTEM1* was expressed at high levels in

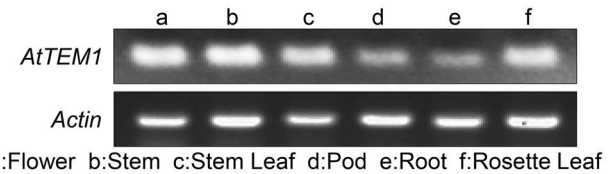


Fig. 1 Spatial expression pattern of *AtTEM1* in *Arabidopsis thaliana*

the flower, stem, rosette and cauline leaf, but relatively low levels in the silique and root tissue.

Sub-cellular localization of AtTEM1 protein

Arabidopsis AtTEM1 is in the RAV family of transcription factors. The main characteristic of RAV members is the presence of two different DNA-binding domains: a B3 and an AP2 domain. The RAV family members have thus been classified as members of either the B3 super-family or the AP2/EREBP (APETALA2) family of transcription factors. By using Clustal v2.1 multiple amino acid alignment and SMART, the putative AP2 and B3 transcriptional activation domains at the N-terminus and the C-terminus were identified. Additionally, the putative nuclear localization signal (NLS) and the B3 repression domain (BRD) were further identified (Fig. S1).

To determine whether the AtTEM1 protein localizes in the nucleus, a CaMV35S::*GFP:AtTEM1* fusion construct was introduced into onion epidermal cells through the *Agrobacterium tumefaciens*-mediated transfection method (Varagona et al. 1992). Compared with the free GFP which was randomly localized in the cytoplasm (Fig. 2d), the *GFP: AtTEM1* was observed predominantly in the nucleus (Fig. 2f, h), indicating that AtTEM1 is a nuclear-localized protein.

AtTEM1 negatively affects flowering time

To determine the role of *AtTEM1* in plant growth and development, reverse genetic analyses were performed. Under LD conditions, *tem1* mutants flowered earlier than WT plants, whereas 35S::*AtTEM1* plants flowered much later than the WT plants. Whereas the *AtTEM1* mutant plants *tem1-1* and *tem1-2* had smaller rosettes size, but the 35S::*AtTEM1* transgenic plants were indistinguishable from the WT plants (Fig. 3a, b). The 35S::*AtTEM1* plants bolted 1.2 weeks later than the WT plants (Fig. 3c, $P < 0.05$) and produced at least 3 extra rosette leaves before flowering (Fig. 3d, $P < 0.05$); however, the *tem1* plant bolted 1.1 weeks earlier than the WT plants (Fig. 3c, $P < 0.05$) but produced nearly the same number of rosette leaves (Fig. 3d, $P < 0.05$) before flowering. The expression of *FT* in the *tem1* and WT plants increased under LD conditions, and the expression of *FT* in the *tem1* was much higher than that in the WT plants; whereas the expression of *FT* in the 35S::*AtTEM1* was almost stable (Fig. 3e). These results confirm that *AtTEM1* acts redundantly to repress *FT* under LD conditions.

Seasonal changes in the day length affect the flowering time of numerous plant species. The flowering of *Arabidopsis* is accelerated by exposure to LD conditions (Castillejo and Pelaz 2008). Compared with the WT plants, the 35S::*AtTEM1* transgenic plants exhibited late-flowering phenotype (Fig. 3), whereas the *tem1* plants were early flowering as reported previously (Castillejo and Pelaz 2008).

AtTEM1 negatively regulates drought tolerance in *Arabidopsis*

To determine the possible involvement of *AtTEM1* in drought and mannitol stress responses, the transpirational water loss rates among the 35S::*AtTEM1, tem1* and WT plants were first compared. The entire aerial shoots were cut from 4-week-old soil-grown plants; and placed on filter paper at ambient temperature; their weights were measured to monitor their water loss over time. After 2 h, 35S::*AtTEM1* plants exhibited a moderate but statistically significant increase in water loss compared with the WT and *tem1* plants. The water loss rate of the 35S::*AtTEM1* transgenic plants was thus higher than that of the WT and *tem1* plants (Fig. 4b), resulting in accelerated plant wilting. Drought treatment was then performed by withholding water for 14 days and then re-watered for further 7 days. After withholding water, the 4-week-old WT and the 35S::*AtTEM1* transgenic plants were discovered to exhibit significant dehydration and wilting whereas most of the *tem1* plants was still alive (Fig. 4a). After re-watering, the *tem1* plants were almost recovered, whereas the 35S::*AtTEM1* plants were almost dead. The same result was obtained from the mannitol treatment of 2-week-old plant seedlings (Fig. 5a) and the seed germination (Fig. 5c). After treatment, only 3.7% of the WT plants and 4.1% ($P < 0.05$) of the 35S::*AtTEM1* plants were survived, but 51.7% ($P < 0.001$) of the *tem1* plants exhibited almost normal growth (Fig. 5b). Under 400 mM mannitol treatment, 58.8% ($P < 0.001$) of *tem1* seeds germinated, while 13.9% of the WT and 15.9% ($P < 0.05$) of the 35S::*AtTEM1* seeds germinated under the stress (Fig. 5d).

Next, whether the physiological indicators changed within the period after the drought was investigated. The REC of electrolyte leakage can be used as an indicator of cell membrane penetrability. By contrast, proline enrichment in stressed plants is a general response to various abiotic stresses and serves as effective indicator of stress tolerance (Akram et al. 2007). MDA, a product of lipid peroxidation, is associated with the oxidative degradation of cell membrane lipids and its abundance serves as an indicator of cell membrane damage.

The REC, MDA content, and proline content revealed that there were no significant differences between the transgenic lines and WT plants before mannitol stress. After the treatment, the REC and MDA content of the *tem1* plants were significantly lower in than those of the WT and 35S::*AtTEM1* plants (Fig. 4c, d). While the highest proline

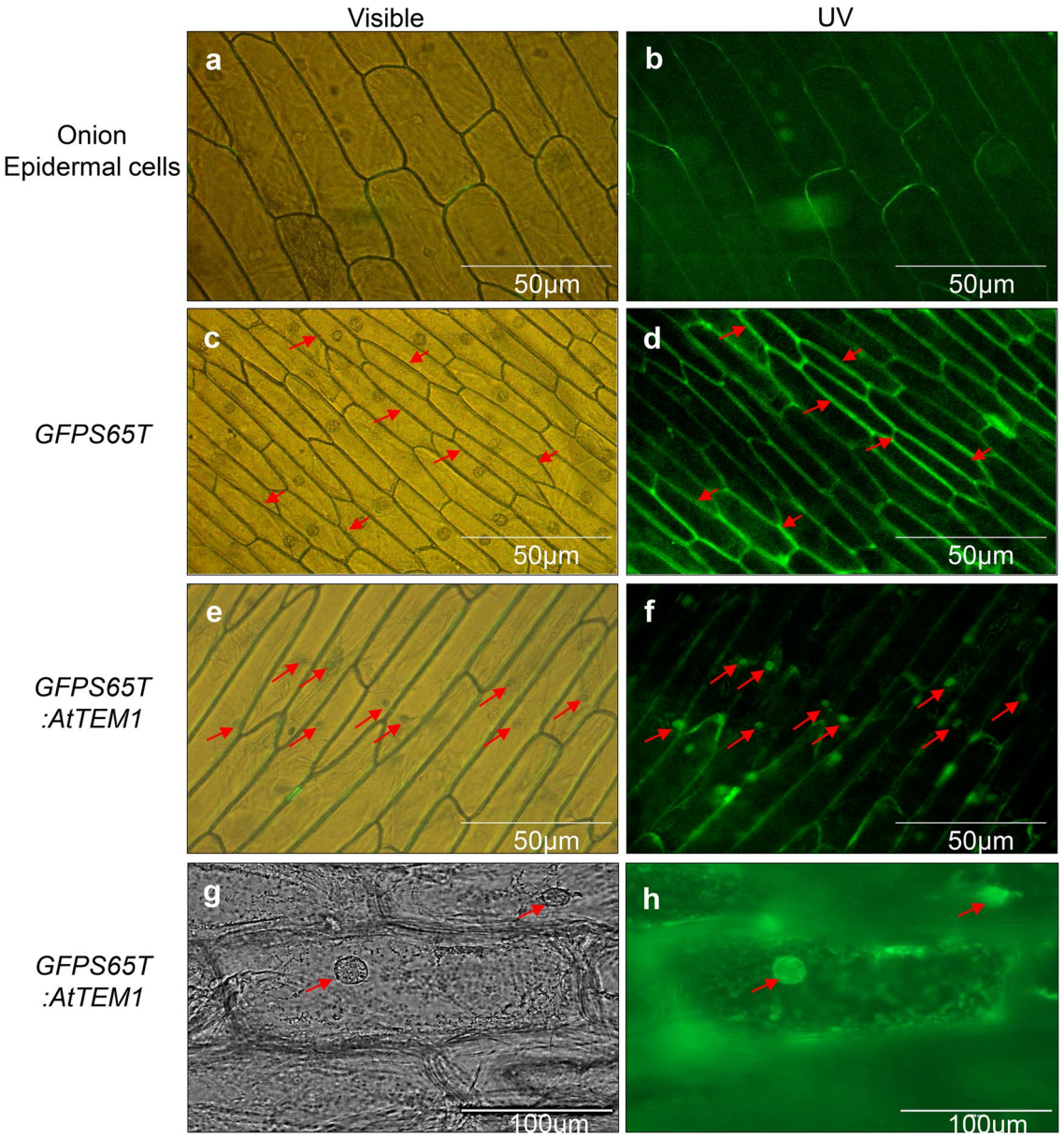
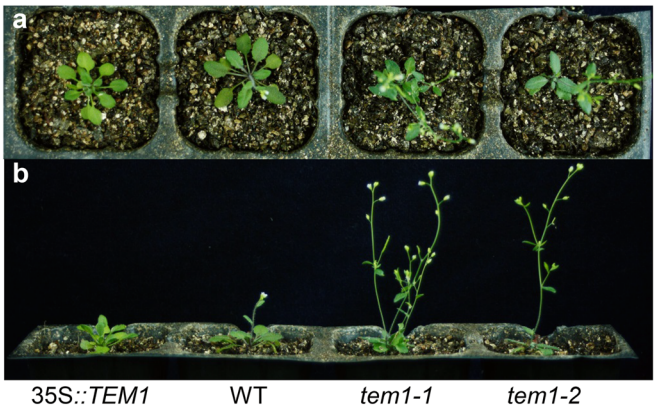


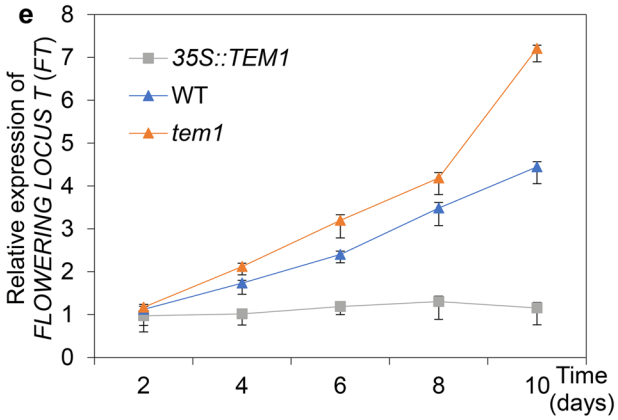
Fig. 2 Subcellular localization of AtTEM1 in onion epidermal cells. **a, b** Onion epidermal cells in visible and ultraviolet light (UV). **c, d** 35S::GFP65T observed in the cytoplasm. **e, f** 35S::GFP65T:*AtTEM1* observed in the nucleus. **g, h** 35S::GFP65T:AtTEM1 observed in the nucleus at 2× amplification. *Scale bars* on panel indicate 50 and 100 μm

accumulation was observed in the *tem1* plants (Fig. 4e). Despite having an enhanced wilting phenotype than the WT plants (Fig. 4a), the 35S::*AtTEM1* transgenic plants were not revealed to have significantly different levels of physiological indicators (Fig. 4c–e).

AtTEM1 negatively affects drought-responsive gene expression

Real-time PCR analysis of *AtTEM1* gene expression demonstrated that *AtTEM1* responded rapidly to the drought stress (Fig. 6a, $P < 0.05$). According to the *FT* expression and growth phenotype results, *AtTEM1* may result in changes in *FT* expression (Fig. 3c, $P < 0.05$). Because 35S::*AtTEM1* transgenic and *tem1* plants had different responses to abnormal drought stress, the expression of drought-responsive genes, namely *PDH, RD22*, and *P5CS*, was investigated. Consistent with their drought tolerant phenotype, these genes were much more strongly induced in *tem1* plants than in the 35S::*AtTEM1* transgenic and WT plants (Fig. 6; $P < 0.05$).





c

Bolting time	
	weeks
35S::*TEM1*	5.60±0.26***
WT	4.40±0.25
tem1	3.29±0.11***

d

Leaf number (mean ±SD)		
	Rosette	Cauline
35S::*TEM1*	15.6±0.21**	3.5±0.11*
WT	12.4±0.34	3.7±0.45
tem1	11.7±0.65**	3.6±0.31*

Fig. 3 Phenotypes and *FT* expression of 35S::*AtTEM1, tem1* and WT *Arabidopsis* under LD conditions. **a, b** 35S::*AtTEM1, tem1* and WT grown under LD conditions for 4 weeks, observed from the *top* (**a**) and from *front* (**b**); **c** *FT* expression; **d** leaf numbers; and **e** bolting time of WT and transgenic plants. *Data marked with asterisk* mean the statistically significant differences between the transgenic and WT plants (*$P<0.05$; **$P<0.01$; ***$P<0.001$)

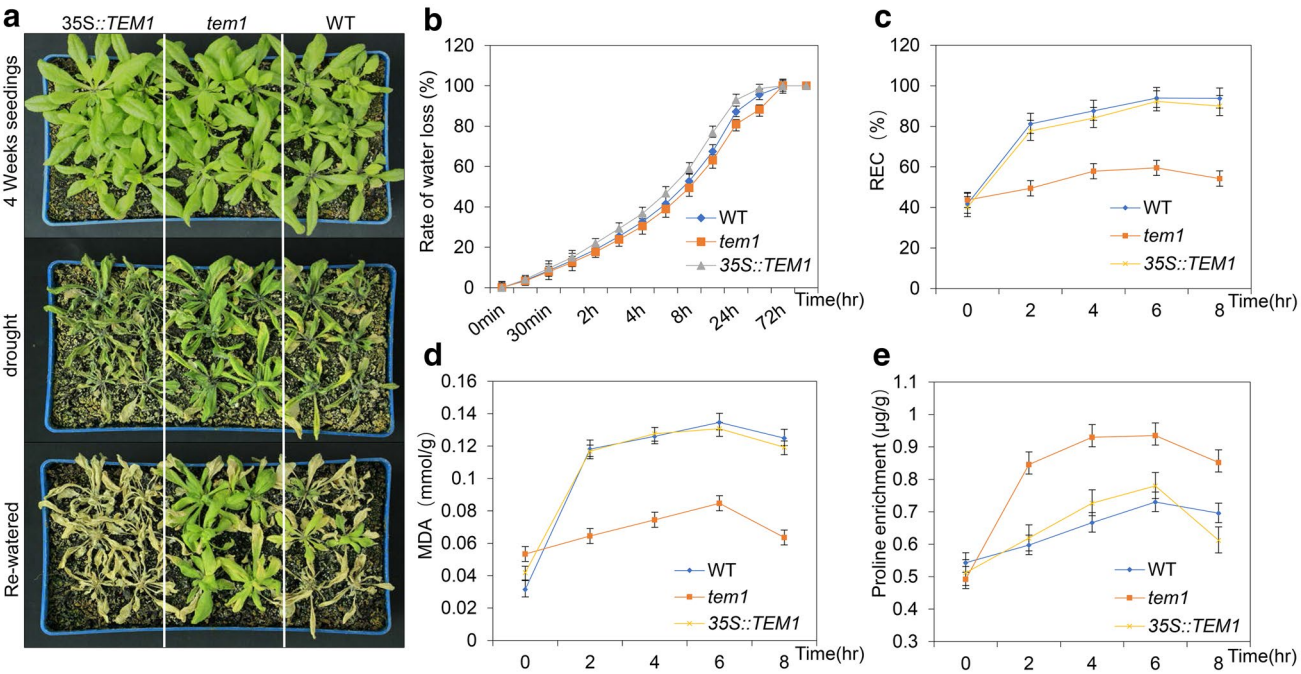
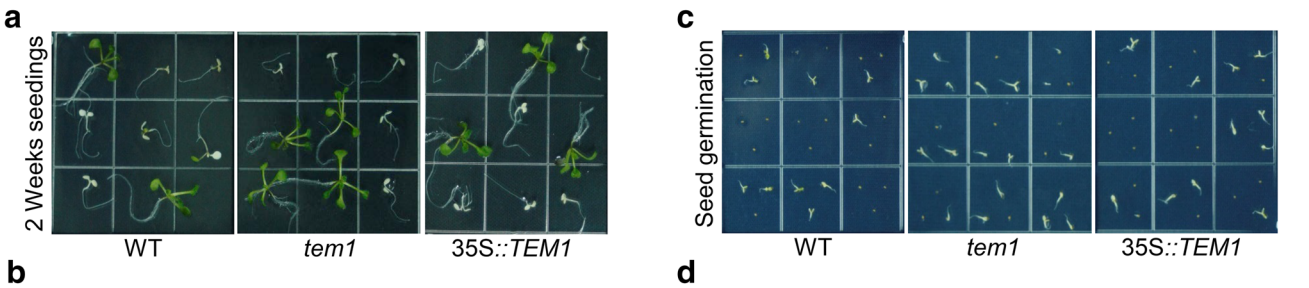


Fig. 4 Phenotypic and biochemical index changes in 35S::*AtTEM1, tem1* and WT *Arabidopsis* under drought treatments. **a** Four-week-old plants before drought treatment (*upper*), after 14 days drought treatment (*middle*) and after re-watering for 7 days (*bottom*); **b** water loss rates of aerial shoots of 4-week-old plants after being exposed to ambient temperature. **c** REC; **d** MDA; **e** proline content



b Seedling survival rate under 400mM mannitol treatment

	Survival rate(%)
35S::*TEM1*	4.1±0.65*
WT	3.7±0.81
tem1	51.7±0.43***

d Seed germination rate under 400mM mannitol treatment

	Seed germination rate(%)
35S::*TEM1*	15.9±0.26*
WT	13.9±0.11
tem1	58.8±0.25***

Fig. 5 Seedling grouth and seed germination under 400 mM mannitol treatment. *Data marked with asterisk* mean the statistically significant differences between the transgenic and WT plants (*$P<0.05$; **$P<0.01$; ***$P<0.001$)

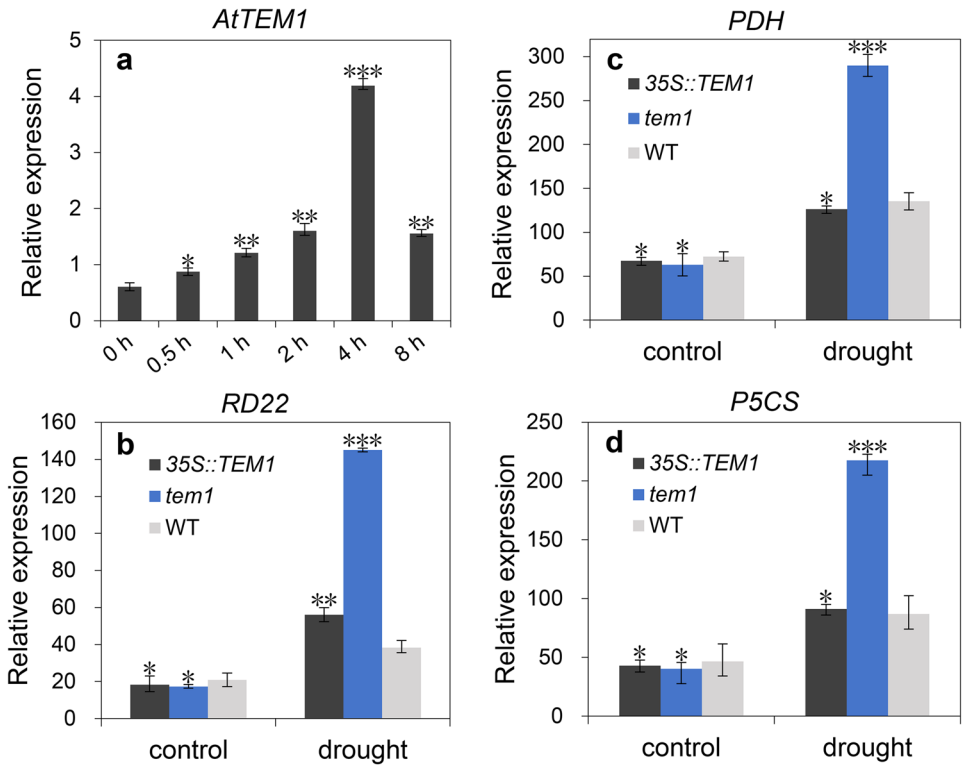


Fig. 6 Expression of *AtTEM1* and drought-inducible genes in WT, 35S::*AtTEM1* and *tem1*. **a** Time course expression level of *AtTEM1* under drought treatment; **b**–**d** expression level of drought responsive gene *RD22* (**b**), *PDH* (**c**) and *P5CS* (**d**) under drought treatment in WT, 35S::*AtTEM1* and *tem1* plants. *Columns marked with asterisk* indicate the statically significant differences between the transgenic and WT plants (*$P<0.05$, **$P<0.01$, ***$P<0.001$)

Discussion

AtTEM1 is an AP2 and B3 domain-containing transcription factor. Down regulation of *AtTEM1* could result in early flowering (Castillejo and Pelaz 2008). In the present study, over-expression of *AtTEM1* in *Arabidopsis* was demonstrated to result in late flowering as was reported previously (Castillejo and Pelaz 2008). This study also demonstrated that AtTEM1 is localized in the nucleus and acts as a negative regulator for flowering time and drought stress response.

AtTEM1 is a versatile regulator of growth and drought stress

In this study, *Arabidopsis* AtTEM1 was demonstrated to be a nuclear localization transcription factor. *AtTEM1* expression was high in the flower, stem, rosette and

cauline leaf, but low in the silique and root tissue. The 35S::*AtTEM1* plants bolted later than the WT plants and produced extra rosette leaves before flowering; however, the *tem1* plants bolted earlier and produced nearly the same number of rosette leaves before flowering. These results suggested that AtTEM1 transcription factor negatively regulates flowering time.

AtTEM1 was also discovered to act negatively on plants under drought stress conditions, more transpirational water loss was observed in the 35S:*AtTEM1* transgenic plants compared with the *tem1* and WT plants (Fig. 4b), and a higher rate of growth resumption was observed in the *tem1* after drought treatment (Fig. 4a). Abiotic stress conditions affect the expression of *RAVs* transcription factors in various species (Fu et al. 2014). Expression of *BnaRAV-1* in *Brassica napus* was induced by treatment with cold, NaCl or polyethylene glycol (PEG) (Zhuang et al. 2011). In present study, the expression of *AtTEM1* transcription factor was induced by drought (Fig. 6a, $P < 0.05$). *PDH, RD22*, and *P5CS* were reported drought-responsive genes (Yamaguchi-Shinozaki and Shinozaki 1993; Kiyosue et al. 1996). The expression of these drought-responsive genes was strongly induced in *tem1* plants than in the WT and 35S:*AtTEM1* transgenic plants, suggesting that *AtTEM1* may negatively regulate the expression of these drought-responsive genes. Under drought and mannitol stresses, increased REC and MDA content and decreased proline content were identified in the 35S:*AtTEM1* plants, while lower REC and MDA content and increased proline content were found in the *tem1* plants (Fig. 4c–e). These results suggested that AtTEM1 in *Arabidopsis* functions as a negative regulator in response to drought and mannitol stresses.

In this study, delayed flowering was discovered in *35S::AtTEM1* transgenic plants. By contrast, early flowering was revealed in *tem1* mutant plants. We also discovered that 35S::*AtTEM1* transgenic plants were less drought tolerant than WT plants, whereas *tem1* mutant plants were much more drought tolerant. Based on these results, AtTEM1 transcription factor is proposed to be a versatile regulator reacting negatively to both flowering time and drought stress.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

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