Mol Biotechnol (2012) 51:160–173
DOI 10.1007/s12033-011-9451-1

RESEARCH

Heterologous Expression of the Chrysanthemum R2R3-MYB Transcription Factor *CmMYB2* Enhances Drought and Salinity Tolerance, Increases Hypersensitivity to ABA and Delays Flowering in *Arabidopsis thaliana*

Hong Shan · Sumei Chen · Jiafu Jiang · Fadi Chen · Yu Chen · Chunsun Gu · Peiling Li · Aiping Song · Xirong Zhu · Haishun Gao · Guoqin Zhou · Ting Li · Xue Yang

Published online: 8 September 2011

Abstract Knowledge on genes related to plant responses to adverse growth conditions and development is essential for germplasm improvement. In this study, a chrysanthemum R2R3-MYB transcription factor gene, designated *CmMYB2* (GenBank accession No. JF795918), was cloned and functionally characterized. Expression of *CmMYB2* in chrysanthemum leaves was up-regulated in response to drought, salinity and cold stress, as well as by treatment with exogenous abscisic acid (ABA). When the gene was constitutively expressed in *Arabidopsis thaliana*, it increased plant sensitivity to ABA and reduced stomatal aperture. Plant survival under drought was improved than in the wild type, as was the plants' salinity tolerance. The level of expression of a number of genes associated with the stress response, including *RD22*, *RD29A*, *RAB18*, *COR47*, *ABA1* and *ABA2*, was raised in the *CmMYB2* transgenic *Arabidopsis* plants. *CmMYB2* transgenic *Arabidopsis* plants were also delayed in flowering. The expression of *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), *LEAFY* (*LFY*) and *APETALA1* (*AP1*) genes involved in flowering was down-regulated in the *CmMYB2* transgenics. Together, these results suggest that *CmMYB2* may be a promising gene for the drought and salt tolerance improvement and flowering-time modulation.

Keywords *Arabidopsis thaliana* · Abiotic stress tolerance · Chrysanthemum · *CmMYB2* · Delayed flowering

H. Shan · S. Chen · J. Jiang · F. Chen (✉) · Y. Chen · C. Gu · P. Li · A. Song · X. Zhu · H. Gao
College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China
e-mail: chenfd@njau.edu.cn

H. Shan · G. Zhou
Institute of Fishery Science of Nanjing, 183 HanZhongmen Street, Nanjing 210036, China

T. Li · X. Yang
College of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China

Introduction

Abiotic stresses, including drought, salt and cold, are among the most serious constraints that inhibit plant growth and development and limit the productivity of plants. The plant tolerance to abiotic stress is a multigenic trait and is mediated by a series of biochemical and physiological reactions with multiple signal transduction and regulations of gene expression [1, 2]. Compared with the functional proteins, the transcription factors always act at the upstream position of the signal transduction and gene regulatory network, which can control a broad range of downstream genes; therefore, can tolerate abiotic stress efficiently. To date, several transcription factors belonging to different transcription factor families, such as MYB, ERF, bZIP and WRKY, have been implicated in the regulation of stress responses [3, 4].

The MYB family is one of the most abundant classes of transcription factors in plants. Their characteristic MYB DNA-binding domain consists of one to three imperfect peptide repeats (R) with 50–53 amino acids, forming the helix-turn-helix motifs [5]. According to the number of peptide repeats in the MYB domain, the MYB family is categorized into 3R-MYB, R2R3-MYB and MYB-related group, respectively. In high plants, R2R3-MYB is the most common type [6, 7]. Variation within certain conserved C-terminal motifs has been used to

classify plant R2R3-MYB genes into 22 subgroups, with members within each subgroup predicted to share similar or identical functions [8]. Numerous R2R3-MYB genes have been found to be involved in the control of plant-specific processes, including primary and secondary metabolism, hormonal signalling, stress responses, cell fate and identity [9–13].

In particular, many R2R3-MYB genes play important roles in the responses to abiotic stresses in *Arabidopsis*. *AtMYB2* can be transiently induced by water stress or high salinity, and functions as a transcriptional activator in abscisic acid (ABA)-inducible gene expression [14, 15]. The *AtMYB15* transcript is regulated by drought and salinity stress, and its over-expression in *A. thaliana* has been shown to improve the level of drought tolerance [16]. High levels of *AtMYB41* expression only occur when plants are stressed by drought, ABA treatment or salinity [17]. *AtMYB44*/*AtMYBR1* (subgroup 22) confers abiotic stresses tolerance partially through ABA-mediated stomatal closure. When treated with ABA, *AtMYB44* transcript accumulation was induced within 30 min, and other abiotic stresses, such as dehydration, salinity and cold, can also activate its expression [18]. In addition, three other members of this subgroup (*AtMYB70*, *AtMYB73* and *AtMYB77*/*AtMYBR2*) are also implicated to be associated with stress responses, *AtMYB73* and *AtMYB77* are induced within 30 min of wounding treatment in *Arabidopsis* [19] and are transiently up-regulated by cold stress [20]. Microarray analysis revealed that the expression of these genes was up-regulated by salt stress treatment in *sos2* (*salt overly sensitive2*) mutants [21].

Delayed flowering is also caused by over-expression of some *MYB* transcription factors in *A. thaliana*. It has been reported that over-expression of EPR1, a novel MYB protein, results in delayed flowering in *Arabidopsis* [22]. Constitutive expression of *CCA1* (encoding a MYB-related transcription factor) in transgenic *Arabidopsis* abolished the circadian rhythm of several genes and caused delayed flowering [23]. In addition, the flowering time of *35S::AtMYB44* transgenic *Arabidopsis* was 6–7 days later than that of wild type plants [18].

Chrysanthemum (*Chrysanthemum morifolium*) is an important ornamental plant worldwide and of wide garden use. So far, no research related to the R2R3-MYB transcription factors in chrysanthemum is available. To discover novel stress-related gene and to better understand the role of R2R3-MYB genes involved in abiotic stress tolerance, here, we isolated a new R2R3-MYB transcription factor *CmMYB2* belonging to the subgroup 22 from chrysanthemum, determined its expression patterns in response to a range of abiotic stresses and gathered some indications of its function by studying the consequences of its heterologous expression in *A. thaliana.*

Materials and Methods

Plants Materials and Treatments

The chrysanthemum variety 'Zhongshanzigui' was obtained from the Chrysanthemum Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. Plants were grown in a 1:1 mixture of garden soil and vermiculite without additional fertilizer, and were maintained in a greenhouse under standard growing conditions. Young plants were watered daily and fertilized with half strength Hoagland's nutrient solution weekly. Various abiotic stress treatments were given to plants at the six-to-eight-leaf stage as follows: For salinity, ABA and mimic drought, the plants were watered with either 200-mM NaCl, 100-μM ABA or 20% polyethylene glycol (PEG) 6000, whereas the cold stress treatment consisted of a period of exposure to 4°C. Totally, 54 plants were included for each treatment and its corresponding control, respectively. The third and fourth leaves counting from the shoot apex were collected from each treated plant. Each experiment contains three biological replicates, and each replicate consisted of a sample of six leaves from three plants at per sampling time point (0, 1, 3, 6, 12 and 24 h), respectively, after the imposition of the stress. The leaves were immediately snap frozen in liquid nitrogen until used to extract RNA.

A. thaliana ecotype Columbia (Col-0) were grown in pots filled with a 1:1:1 mixture of perlite:vermiculite:soilrite in a growth chamber (SANYO, MLR-350H, Japan) with a 16 h, 23°C lit (80–100 μmol/m^2/s illumination) and an 8 h, 18°C unlit regime, and watered every 4 days. To determine the expression level of stress-responsive genes in *CmMYB2* transgenics, 3-week-old Col-0 and transgenic *A. thaliana* plants were treated with 250-mM NaCl and the rosette leaves were harvested at the indicated times (0, 1, 3, 6, 12 and 24 h), then stored at −80°C till use. To determine the expression level of flowering-time genes in *CmMYB2* transgenics, 14-day-old Col-0 and transgenic *A. thaliana* seedlings were used for RNA extraction and expression analysis.

Full-Length cDNA Cloning and Sequencing

Total RNA was isolated from chrysanthemum leaves using the RNAiso reagent (TaKaRa, Japan), following the manufacturer's instructions. The cDNA first strand was synthesized from 1 μg total RNA using SuperScript III reverse transcriptase (Invitrogen, USA), according to the manufacturer's instructions. A gene-specific primer pair (F and R) was designed to amplify a fragment of *CmMYB2* based on the sequence of a chrysanthemum EST [24], and RACE PCR was then used to obtain the full-length cDNA. For the 3′ RACE reaction, the first-strand cDNA was synthesized

using an oligo (dT) primer incorporating the sequence of the adaptor primer. Then, a nested PCR using the gene-specific primer pair GSP3′-1/-2 and the adaptor primer (dT-AP) was employed. For the 5′ RACE, the nested PCR used the 5′ RACE adaptor primer (Abridged Anchor Primer, AAP), the Abridged Universal Amplification Primer (AUAP) provided with the 5′ RACE System kit v2.0 (Invitrogen) and the gene-specific primer pair (GSP5′-531, GSP5′-299 and GSP5′-167). PCR products were purified with a Biospin Gel Extraction kit (Bio Flux) and cloned into pMD19-T easy (TaKaRa) for sequencing. Finally, a pair of gene-specific primers (Full-F and Full-R) was designed from the putative 5′ and 3′ UTR sequences, and these were used to amplify the complete *CmMYB2* open reading frame (ORF). The sequences of all the above primers are given in Table 1. Chrysanthemum genomic DNA was isolated from young leaves using a CTAB method [25], and the genomic copy of *CmMYB2* was amplified using the primer pair Full-F/Full-R. The resulting product was purified using a Biospin Gel Extraction kit and cloned into pMD19-T easy for sequencing.

Sequence Alignment and Phylogenetic Analysis of *CmMYB2*

The CmMYB2 amino acid sequence was aligned with its homologues using ClustalW (http://www.ebi.ac.uk/clustalW/) [26]. A neighbour-joining-based phylogenetic tree was constructed using BioEdit and TreeView software [27].

Quantitative Real-Time PCR (qRT-PCR)

To determine the expression pattern of *CmMYB2* in chrysanthemums under various abiotic stress treatments, total RNA was extracted from frozen leaves using the RNAiso reagent (TaKaRa, Japan); putative genomic DNA contamination was removed by DNaseI treatment. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, USA), and quantitative real-time PCR (qRT-PCR) was performed using SYBR® Green I (TOYOBO, Japan) in Rotor-Gene 3000 (Corbett, Australia). The primer pair MYB2-RT-F/-R (Table 1) was applied to amplify a 124-bp fragment in the 3′ region of the gene, avoiding the more well-conserved segments of the gene. The chrysanthemum *GAPDH* gene (*CmGAPDH*, GenBank accession number: DK941612) was used as a reference. Each 25 µl qRT-PCR contained 10 µl SYBR Green PCR master mix, 0.2 µM of each primer and 10 ng cDNA, and the amplification regime consisted of an initial denaturation of 95°C/60 s, followed by 40 cycles of 95°C/15 s, 60°C/15 s and 72°C/45 s. The resulting data are represented by the means ± SD of three replicates. Relative expression levels were calculated by the $2^{-\triangle\triangle CT}$ method, where $\triangle\triangle CT = (CT, \text{Target} - CT, \text{GAPDH})\text{Time}_x - (CT, \text{Target} - CT, \text{GAPDH})\text{Time}_0$ [28]. The data were scaled by setting the expression of *CmMYB2* in untreated leaves at 0 h as 1.

Ten stress-responsive genes were *RD22*, *RD29A*, *RAB18*, *COR47*,*COR15*, *ABA1*, *ABA2*, *ABI1*, *HAB1* and *HAB2*, and the six flowering-related genes were *CO*, *FLC* (*FLOWERING LOCUS C*), *FT*, *LFY*, *SOC1* and *Ap1*. *Arabidopsis AtUBQ* gene (GenBank accession number: NM_116771.5) was used as the reference. The sequences of all relevant primers are listed in Table 1. To analyse stress-responsive gene expression, cDNA was synthesized using RNA from non-transgenic and *CmMYB2*-expressing *A. thaliana* subjected to salt stress as template. For flowering-related gene expression analysis, cDNA was synthesized using RNA from non-transgenic and *CmMYB2*-expressing *A. thaliana* as template. Quantitative RT-PCR analysis was performed as described above.

Transgene Construction and *A. thaliana* Transformation

The coding sequence of *CmMYB2* cDNA was amplified using a forward primer (MYB2-1301-F) incorporating a *Bam*HI restriction site and a reverse primer (MYB2-1301-R) engineered to include a *Sac*I cleavage site (Table 1). The *Bam*HI–*Sac*I-digested amplicons were inserted into pCAMBIA1301 driven by the CaMV 35S promoter to generate a *35S::CmMYB2* construct, which was introduced into Col-0 through an *Agrobacterium tumefaciens* EHA105-mediated floral dip method [29]. Transformed progeny were selected by germination on MS medium containing 50 µg/ml hygromycin, and these were advanced by self-pollination to obtain the T_2 generation, when RT-PCR analysis was used to confirm their transgenic status.

Measurement of Stomatal Aperture in Detached *A. thaliana* Leaves

Before treating with ABA, rosette leaves of 3-week-old *Arabidopsis* plants were detached and floated abaxial side down in the stomata-opening solution (50-mM KCl, 10-µM $CaCl_2$ and 10-mM MES, pH 6.15) [30] for 12 h at a relative humidity of 95%. Stomatal aperture was assessed under a light microscope 1.5 h after the addition of either 0-, 1- or 10-µM ABA [31].

Stress Tolerance Assay of *A. thaliana* Plants Expressing *CmMYB2*

A triplicated germination assay comprising ~100 seeds each per replicate of Col-0 and the transgenics was carried out on MS medium containing various concentrations of

Table 1 Primer names and sequences used in this study

Primer name	Sequences
F	5′-GTCCAGACGAGGACGAAATGT-3′
R	5′-GTCTTATCGGCATCACATTCG-3′
Oligo(dT)	5′-GACTCGAGTCGACATCGATTTTTT TTTTTTTTTTT-3′
dT-AP	5′-GACTCGAGTCGACATCGA-3′
GSP3′-1	5′-CTTTTCACCGTCGTTCCTCCT-3′
GSP3′-2	5′-GCGAATGTGATGCCGATAAGA-3′
AAP	5′-GGCCACGCGTCGACTAGTACGGGIIG GGIIGGGIIG-3′
AUAP	5′-GGCCACGCGTCGACTAGTAC-3′
GSP5′-531	5′-AGAAATGGGTACGCCTGAAC-3′
GSP5′-299	5′-AAAGCCCTATGCTCCACTTC-3′
GSP5′-167	5′-AACATTTCGTCCTCGTCTGG-3′
Full-F	5′-CATCCTCTCATTTTCTTTCCAA-3′
Full-R	5′-CTACCCTCATCATATCCCATCTC-3′
MYB2-RT-F	5′-AGAAGCCGTTTGTGCCGTTTAG-3′
MYB2-RT-R	5′-CCGTTACACATTCCGTTACCAC-3′
CmGAPDH-F	5′-CTGCTTCTTTCAACATCATTCC-3′
CmGAPDH-R	5′-CTGCTCATAGGTAGCCTTCTTC-3′
RD22F	5′-ACTTGGTAAATATCACGTCAGGGCT-3′
RD22R	5′-CTGAGGTGTTCTTGTGGCATACC-3′
RD29AF	5′-GATAACGTTGGAGGAAGAGTCGG-3′
RD29AR	5′-TCCTGATTCACCTGGAAATTTCG-3′
RAB18F	5′-GCAGTATGACGAGTACGGAAATCC-3′
RAB18R	5′-CCTTGTCCATCATCCGAGCTAGA-3′
COR47F	5′-ATGGCTGAGGAGTACAAGAACAACGTT-3′
COR47R	5′-TCTTCTTCTTCTTCTCCTTCTTTTCCT-3′
COR15AF	5′-GTGACGGATAAAACAAAAGAGG-3′
COR15AR	5′-GACCCTACTTTGTGGCATCCTT-3′
ABA1F	5′-GCTATGAAGGTGATCTGCTTGTGG-3′
ABA1R	5′-TTCATACCATTTGGAGCATCAGC-3′
ABA2F	5′-ATTGATCACTGGAGGAGCCACAG-3′
ABA2R	5′-ATTACGAATATCAGGGCACGGTG-3′
ABI1F	5′-AGCTGCTGATATAGTCGTCGTTGATA-3′
ABI1R	5′-GAGGATCAAACCGACCATCTAACA-3′
HAB1-F	5′-TTCTCGCCATGTCTAGGTCCAT-3′
HAB1-R	5′-TTCGCAGACTTCTTGGTTGTTC-3′
HAB2-F	5′-GCAGAAGTCCTTATTGCGAGTC-3′
HAB2-R	5′-TTCATAAACACTCCTGCTTCCT-3′
CO-F	5′-TACAGAGAGAAGAGGAAGACAAGG-3′
CO-R	5′-GGAGTATCAGAATGAAGGAACAAT-3′
FT-F	5′-ACTAATGGCTTGGATCTAAGGC-3′
FT-R	5′-ATATTCTCGGAGGTGAGGGTTG-3′
LFY-F	5′-CGAAGAAATCAGGAGCGAGTTA-3′
LFY-R	5′-TAGGCAGTGGAGAGCGTAACAG-3′
SOC1-F	5′-AAAACGAGAAGCTCTCTGAAAAGT-3′
SOC1-R	5′-GAACAAGGTAACCCAATGAACAAT-3′
AP1-F	5′-GTTGCTCTTGTTGTCTTCTCCC-3′
AP1-R	5′-CTCCATCGACCAGTTTGTATTG-3′
AtUBQ-F	5′-AGGACAAAGAGGGTATCCCA-3′
AtUBQ-R	5′-CAGACGCAAGACCAAGTGAA-3′
MYB2-1301-F	5′-GGATCCATGGCTACTGTTTCGAAA-3′
MYB2-1301-R	5′-CGAGCTCCTAGTCTATTTTACTAATCCC-3′

ABA (0, 0.1, 0.5, 1, 3 and 5 μM) or NaCl (0, 100, 150 and 200 mM). Dormancy was first broken by holding imbibed seed in the dark for 48 h at 4°C, before removing them to a growth chamber (16 h, 23°C lit [80–100 μmol/m^2/s illumination] and 8 h, 18°C unlit regime) for 10 days, at which point successfully germinated seed were taken as those whose radicle had reached a length of 1 mm. Germination rate was calculated as the percentage of the original number of seeds plated.

For the salinity tolerance test, 3-week-old Col-0 and *CmMYB2* transgenic *Arabidopsis* plants were watered for 12 days at 4-days intervals, first with 100-mM, then with 200-mM and finally with 300-mM NaCl after which their survival rate was calculated.

For drought tolerance, water was withheld for 14 days from 2-week-old Col-0 and *CmMYB2* transgenic *Arabidopsis* plants grown under sufficient moisture, and their appearance and survival rate scored 7 days after being re-watered.

For the measurement of water loss, ten fully expanded rosette leaves were detached from 3-week-old Col-0 and *CmMYB2* transgenic *Arabidopsis* plants, weighed and then allowed to dry at 25°C, 60% relative humidity. Their weight was recorded at 30-min intervals and the water loss was measured by the fresh-weight loss of the detached leaves.

Results

The Sequence of *CmMYB2*: A Chrysanthemum R2R3-MYB Transcription Factor

According to the EST libraries of *Chrysanthemum*, two MYB genes were isolated by RT-PCR and RACE methods, which were designated as *CmMYB1* and *CmMYB2* (GeneBank accession No. JF795917 and JF795918). *CmMYB1* was characterized as a transcription repressor regulating lignin biosynthesis and discussed in another paper (unpublished). Here, we focus on the molecular characterization of *CmMYB2*. The full-length *CmMYB2* cDNA consisted of 1,331 nucleotides, comprising a 948-bp ORF encoding 315 residues. The predicted gene product is a protein of molecular mass 34.6 kDa and a pI of 8.87. The

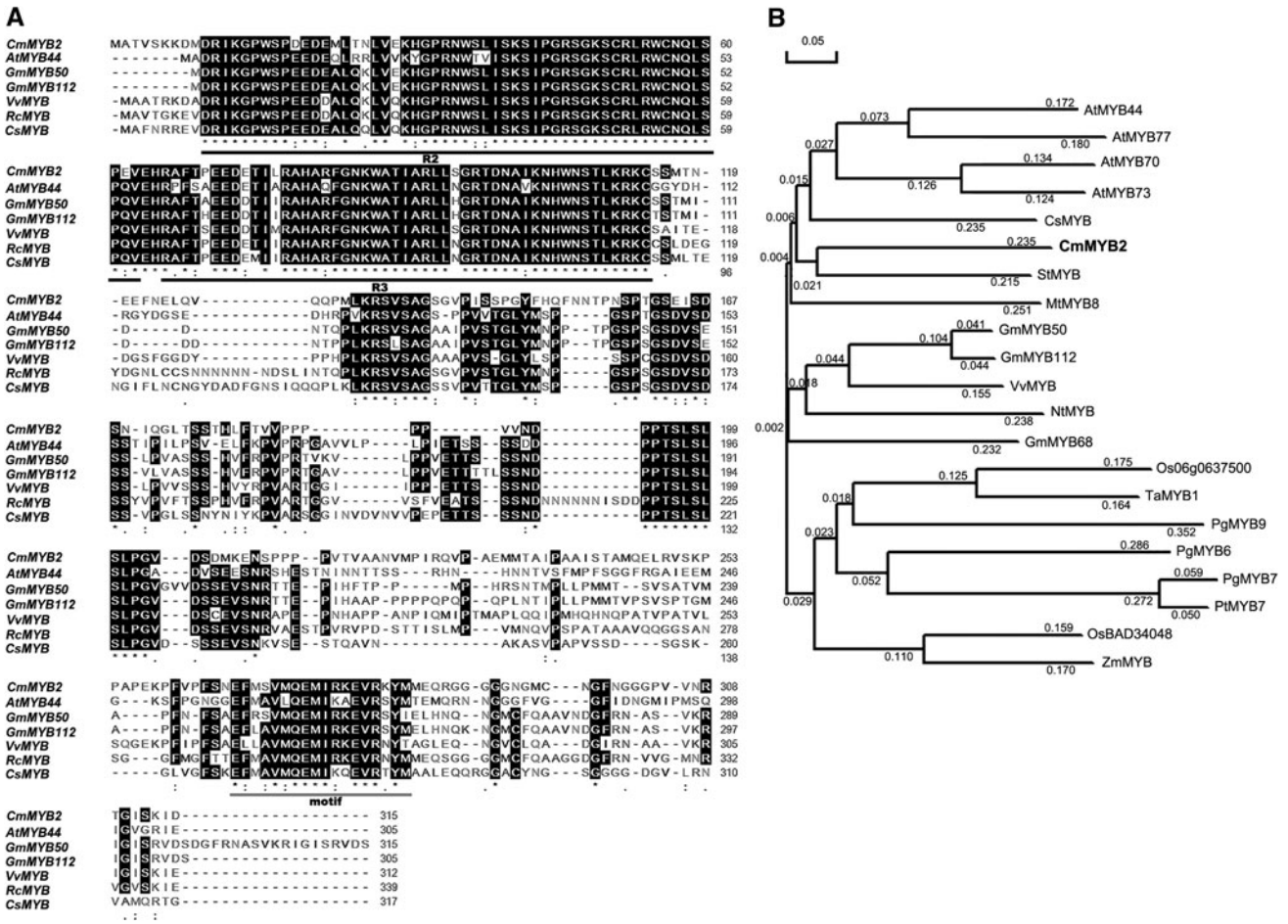


Fig. 1 The deduced peptide sequences of CmMYB2 and other MYBs. **a** Alignment of CmMYB2 with homologous proteins. The R2 and R3 MYB repeats are *underlined in black*, and the (GxFMxVVQEMIxxEVRSYM) motif in *grey*; **b** Phylogeny of CmMYB2 and related MYBs. The phylogenetic tree file was produced by BioEdit and TreeView software. Bootstrap values indicate the distance of each branch and the scale bar represents 0.05 substitutions per site. The genes encoding the amino acid sequences and their GenBank accession numbers are the following: AtMYB44 (XP_002866710), AtMYB77 (NP_190575), AtMYB70 (NP_179910), AtMYB73 (NP_195443), CsMYB (ABQ10816), StMYB (AAG08959), MtMYB8 (ABR28330), GmMYB50 (ABH02824), GmMYB112 (ABH02852), VvMYB (AAY28930), NtMYB (BAC53938), GmMYB68 (ABH02831), Os06g0637500 (NP_001058148), TaMYB1 (ABC86569), PgMYB9 (ABQ51225), PgMYB6 (ABQ51222), PgMYB7 (ABQ51223), PtMYB7 (ABD60281), OsBAD34048 (BAD34048), ZmMYB (NP_001151336). CmMYB2 is in *bold*

sequence includes an R2R3 MYB domain with two DNA-binding sites, one between residues 8 and 63, the other between residues 65 and 114. Its level of sequence similarity with GmMYB50, GmMYB112, VvMYB, CsMYB, RcMYB and AtMYB44 was, respectively, 57, 56, 56, 56, 55 and 53 (Fig. 1a). A comparison with a set of full-length MYB proteins from other species clustered CmMYB2 with the *A. thaliana* R2R3-MYB subgroup 22 members AtMYB44, AtMYB77, AtMYB70 and AtMYB73 (Fig. 1b). In addition, just as in AtMYB44, CmMYB2 includes the characteristic motif GxFMxVVQEMIxxEVRSYM in the C-terminal part (Fig. 1a), suggesting *CmMYB2* can be classified into the 22 subgroup [8]. The alignment of the cDNA and genomic DNA copies of *CmMYB2* indicated that the gene is intron-free (data not shown).

The Expression of *CmMYB2* Is Induced by Abiotic Stress

CmMYB2 transcript abundance increased rapidly in response to mimic drought, salinity or cold stress within 1 h of the stress being imposed, whereas in the absence of stress, the gene was not detectably expressed. Its expression peaked within 3 h of PEG being added to the medium, and then gradually decreased (Fig. 2a). When treated with 200-mM NaCl, its expression level increased gradually until 24 h after the treatment (Fig. 2b). *CmMYB2* expression was also induced by low temperature, peaking 1 h after the beginning of the cold treatment and thereafter declining to a stable level by 6 h post the imposition of the cold treatment (Fig. 2c). *CmMYB2* transcript abundance

peaked 3 h after the plants had been exposed to 100-μM ABA, before falling back during subsequent hours (Fig. 2d).

The Effect on *A. thaliana* of the Constitutive Expression of *CmMYB2*

The two selected *A. thaliana* transgenic lines (*35S:: CmMYB2-1* and *-2*) fully expressed the transgene (Fig. 3a). The flowering time of the *CmMYB2* transgenics under long day conditions was later than that of Col-0 (Fig. 3b), with the latter reaching flowering at 36.5 days after sowing (DAS), and the former at 42.8 and 43.6 DAS (Fig. 3c). At the time of flowering, the two transgenic lines had formed a mean of 19.2 ± 1.6 and 19.9 ± 1.3 rosette leaves, compared with 11.8 ± 0.79 in Col-0 (Fig. 3d). To investigate the possible reasons responsible for the late flowering of *CmMYB2* transgenics, the expression of flowering-time genes was analysed by qRT-PCR. Among the flowering-time genes investigated, the expression of *CO*, *FT*, *SOC1*, *LFY* and *AP1* was lower in the *CmMYB2* lines than in Col-0 (Fig. 3e, g–j). However, the expression of *FLC* was not changed (Fig. 3f).

Fig. 3 The growth of *CmMYB2* transgenics and Col-0 plants. **a** RT-PCR demonstrating the heterologous expression of *CmMYB2* in *A. thaliana*. The *AtUBQ* sequence was used as an internal control; **b** The transgenics flowered later than Col-0; **c** Flowering time of the *CmMYB2* transgenics and Col-0. Flowering was taken to be when the main inflorescence shoot had reached a height of 1 cm. Values represent the mean of 15 plants; **d** The number of rosette leaves at flowering. Values represent the mean of 15 plants; **e–j** The expression of flowering-related genes in *CmMYB2* transgenics and Col-0. The seedlings of 14-day-old Col-0 and *CmMYB2* transgenic *Arabidopsis* were used for RNA extraction, and expression level was determined by qRT-PCR. Mean values and standard deviation were calculated from triplicated assays

In the absence of exogenously supplied ABA, the *CmMYB2* transgenics germinated as freely as Col-0. However, the germination of the *CmMYB2* transgenics was inhibited more severely in the presence of 0.5–3.0-μM ABA in the germination medium (Fig. 4a). At 0.5-μM ABA, the germination rate of the two *CmMYB2* transgenics fell to 65.0 and 62.6%, whereas that of Col-0 was 88.2%. At 3-μM ABA, only 35.4 and 31.5% of the transgenic seed germinated, whereas 68.6% of Col-0 seed was able to do so. The presence of ABA also resulted in the *CmMYB2* transgenics having a higher rate of stomatal closure than Col-0 plants (Fig. 4b). Treatment with 1-μM ABA reduced

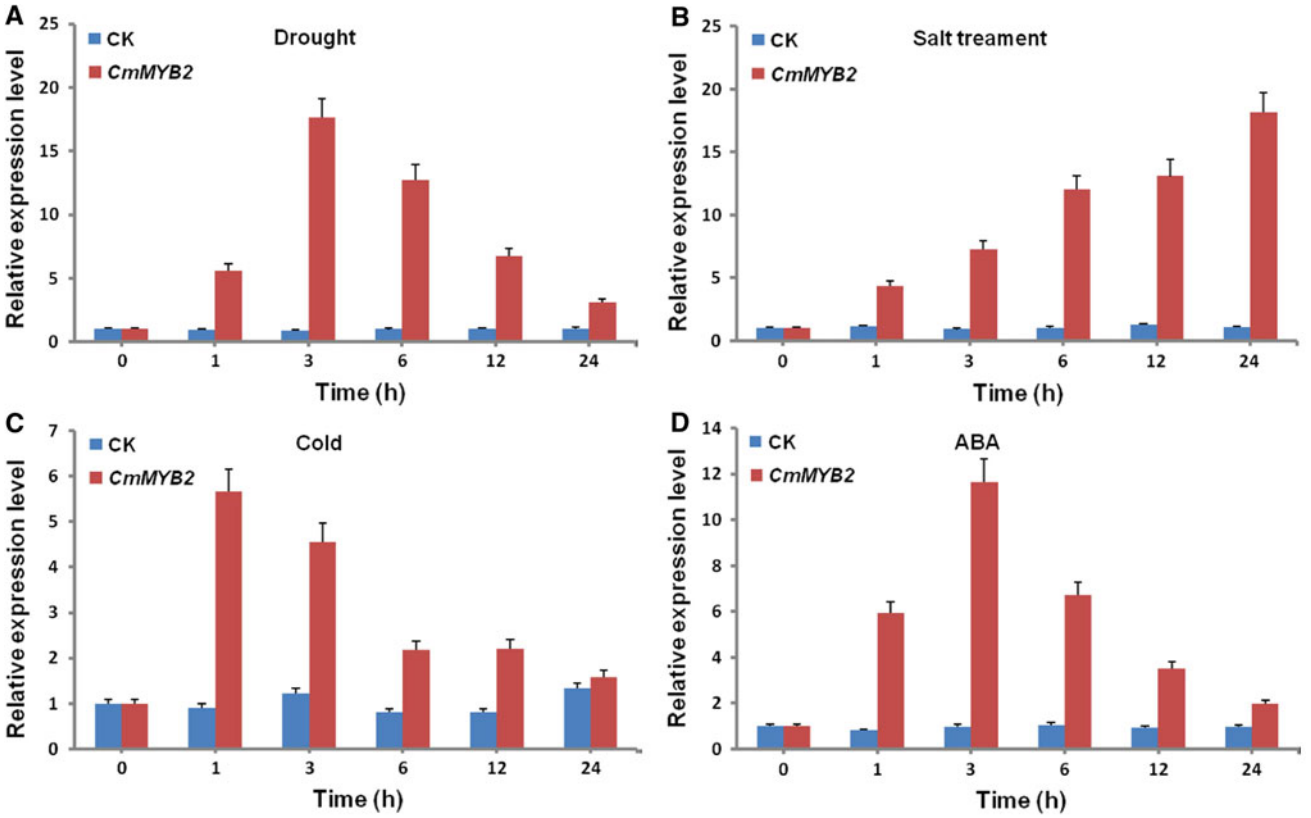
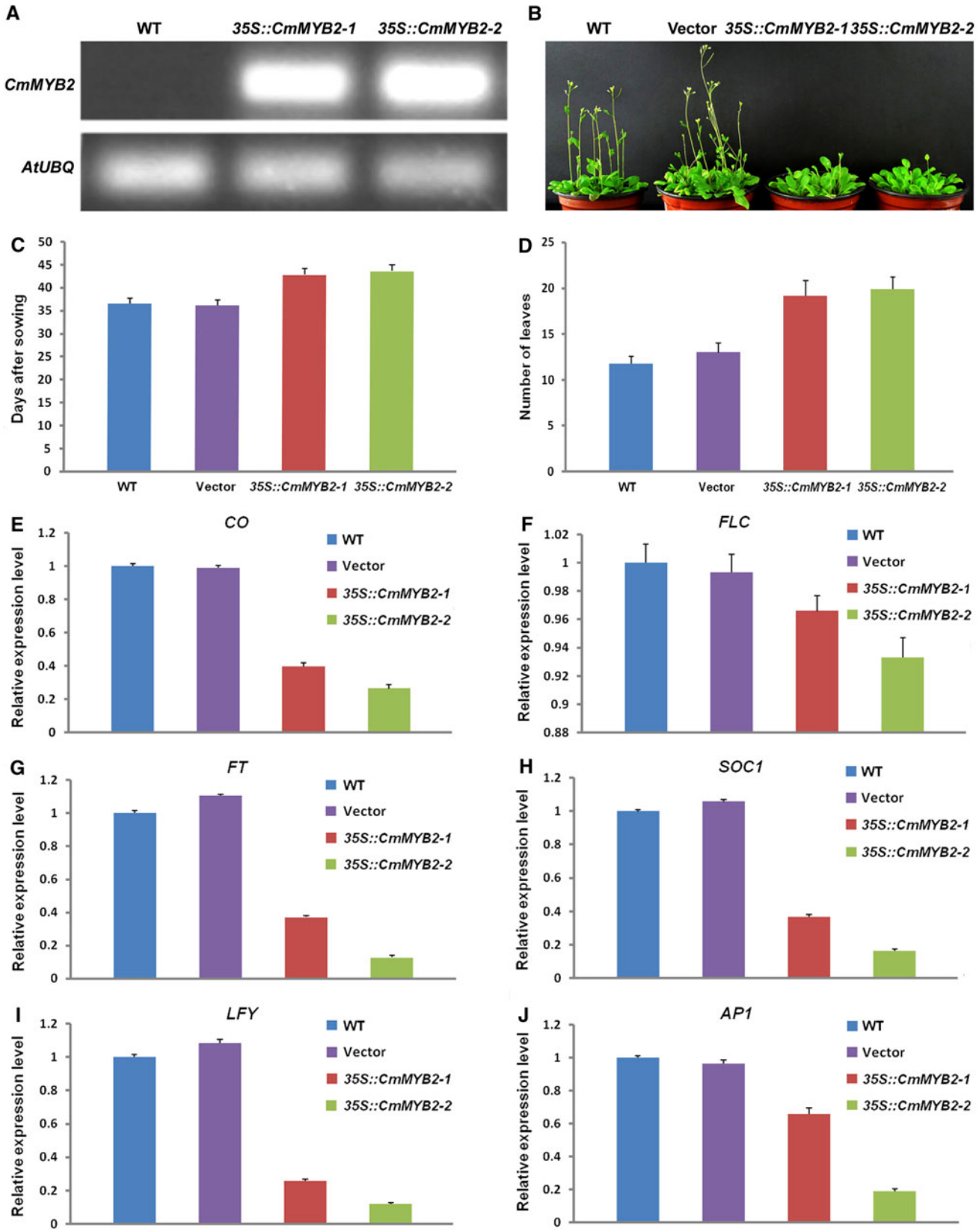


Fig. 2 *CmMYB2* expression profiles in chrysanthemum plants subjected to various stresses, as assayed by qRT-PCR. Mean values and standard deviation calculated from triplicated assays. The relative expression of the *CmMYB2* gene in untreated leaves was used as *CK*. **a** Drought stress (20% PEG 6000), **b** Salinity stress (200-mM NaCl), **c** Cold stress (4°C) and **d** Treatment with 100-μM exogenous ABA


A
WT
35S::CmMYB2-1
35S::CmMYB2-2
CmMYB2
AtUBQ
B
WT
Vector
35S::CmMYB2-1
35S::CmMYB2-2
C
Days after sowing
WT
Vector
35S::CmMYB2-1
35S::CmMYB2-2
D
Number of leaves
WT
Vector
35S::CmMYB2-1
35S::CmMYB2-2
E
CO
Relative expression level
WT
Vector
35S::CmMYB2-1
35S::CmMYB2-2
F
FLC
Relative expression level
WT
Vector
35S::CmMYB2-1
35S::CmMYB2-2
G
FT
Relative expression level
WT
Vector
35S::CmMYB2-1
35S::CmMYB2-2
H
SOC1
Relative expression level
WT
Vector
35S::CmMYB2-1
35S::CmMYB2-2
I
LFY
Relative expression level
WT
Vector
35S::CmMYB2-1
35S::CmMYB2-2
J
AP1
Relative expression level
WT
Vector
35S::CmMYB2-1
35S::CmMYB2-2

stomatal aperture in Col-0 to 84.1% of the control, but the reduction in the *CmMYB2* transgenics was ~60% of the control. At 10-μM ABA, Col-0 plants retained a ~60% level of stomatal aperture, but that of the two *CmMYB2* transgenics fell to, respectively, 34.7 and 31.0%.

The ability to germinate in the presence of <100-mM NaCl was similar in the Col-0 and *CmMYB2* transgenic *Arabidopsis* plants. However, when the salt concentration was raised to 150 mM, the germination rate of Col-0 fell to just 28.8% while those of the two *CmMYB2* transgenics were, respectively, 75.9 and 83.3%. At 200-mM NaCl, <3% of the Col-0 seeds germinated, but 16.7% of the *35S::CmMYB2-1* and 12.5% of the *35S::CmMYB2-2* seeds continued to do so (Fig. 5a, b). When the plants were challenged with salinity, the *CmMYB2* transgenics retained their turgor and continued to grow, whereas Col-0 plants rapidly wilted. At 300-mM NaCl, the survival rates of the two *CmMYB2* transgenics were 92 and 90.4%, but only 31.1% of Col-0 plants could survive this level of salinity (Fig. 5c).

The *CmMYB2* transgenics also showed an improved level of drought tolerance over Col-0 (Fig. 6a). Their survival rates (86.5 and 78.6%) were far higher than that of Col-0 (19.8%). In the detached leaf test used to assess the rate of water loss, Col-0 plants lost 40% of their water within 3 h, whereas the two *CmMYB2* transgenics lost, respectively, 27.9 and 29.3% (Fig. 6b).

The Expression of Various Stress Responsive Genes was Enhanced in the Transgenic Lines Expressing *CmMYB2*

The expression of six genes (*RD22*, *RD29A*, *RAB18*, *COR47*, *ABA1* and *ABA2*) was strongly induced in the *CmMYB2* transgenics both under normal growing conditions and in the presence of 250-mM NaCl (Fig. 7a–d, f, g). That of *ABI1*, *HAB1*, *HAB2* and *COR15* was lower in the *CmMYB2* transgenics than in Col-0 (Fig. 7e, h–j). To our surprise, an increase of *CmMYB2* mRNA level was also observed (Fig. 7k), because the expression of *CmMYB2* in *Arabidopsis* was driven by the 35S promoter, which is not an inducible promoter, it is likely that stress treatment could stabilize *CmMYB2* mRNA.

Discussion

Inducible Expression of *CmMYB2* in Chrysanthemum by Abiotic Stresses and ABA

MYB transcription factor genes constitutes one of the most abundant regulatory gene families in plants, and the subfamily containing two repeats of the R2R3 DNA-binding domain is the largest in the higher plants. Here, we have shown that *CmMYB2*, which on the basis of its sequence and phylogeny can be classified into R2R3-MYB subgroup 22. Within this subgroup, *AtMYB77*, *AtMYB73* and *AtMYB44* were shown to be associated with abiotic stress responses [18, 20, 21]. With no exception, *CmMYB2* expression in leaves was strongly induced subjected to drought, salinity and cold stress, as well as by treatment with exogenous ABA. Many R2R3-MYB genes induced by this combination of stress factors are also inducible by the exogenous application of ABA (e.g., *AtMYB2* [15], *AtMYB15* [16] and *AtMYB102* [32]).

CmMYB2 Delayed Flowering and Decreased the Expression of Flowering Time Genes

In *Arabidopsis*, the flowering time is regulated by at least four genetic pathways: photoperiod, vernalization, autonomous and gibberellin (GA)-dependent pathways [33].

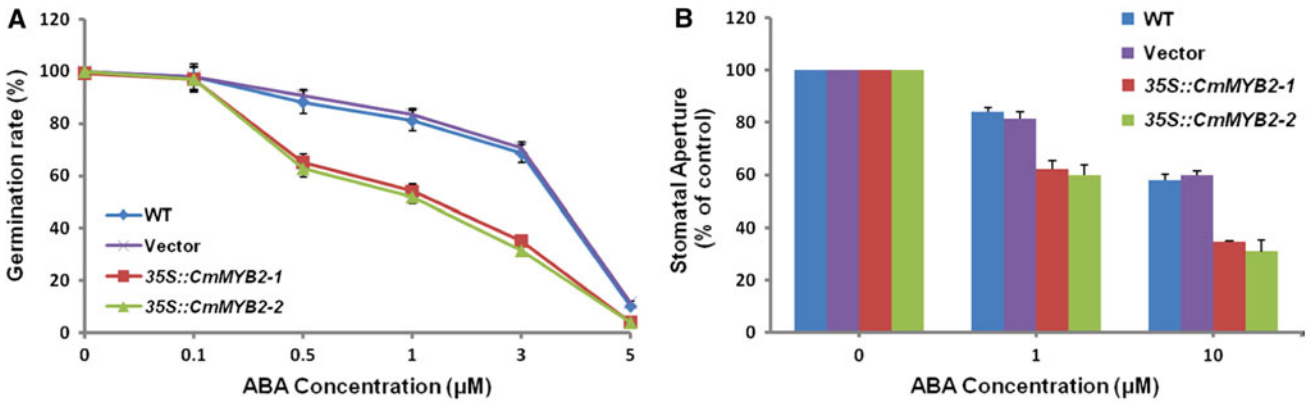


Fig. 4 Responses of *CmMYB2* transgenic *Arabidopsis* plants to the application of exogenous ABA. **a** Germination rates of Col-0 and *CmMYB2* transgenic seeds in the presence of various concentrations of ABA. Means calculated from triplicated germination tests. **b** Stomatal aperture in leaves of Col-0 and the two *CmMYB2* transgenics treated with 1 or 10-μM ABA. Values shown are the means from three experiments

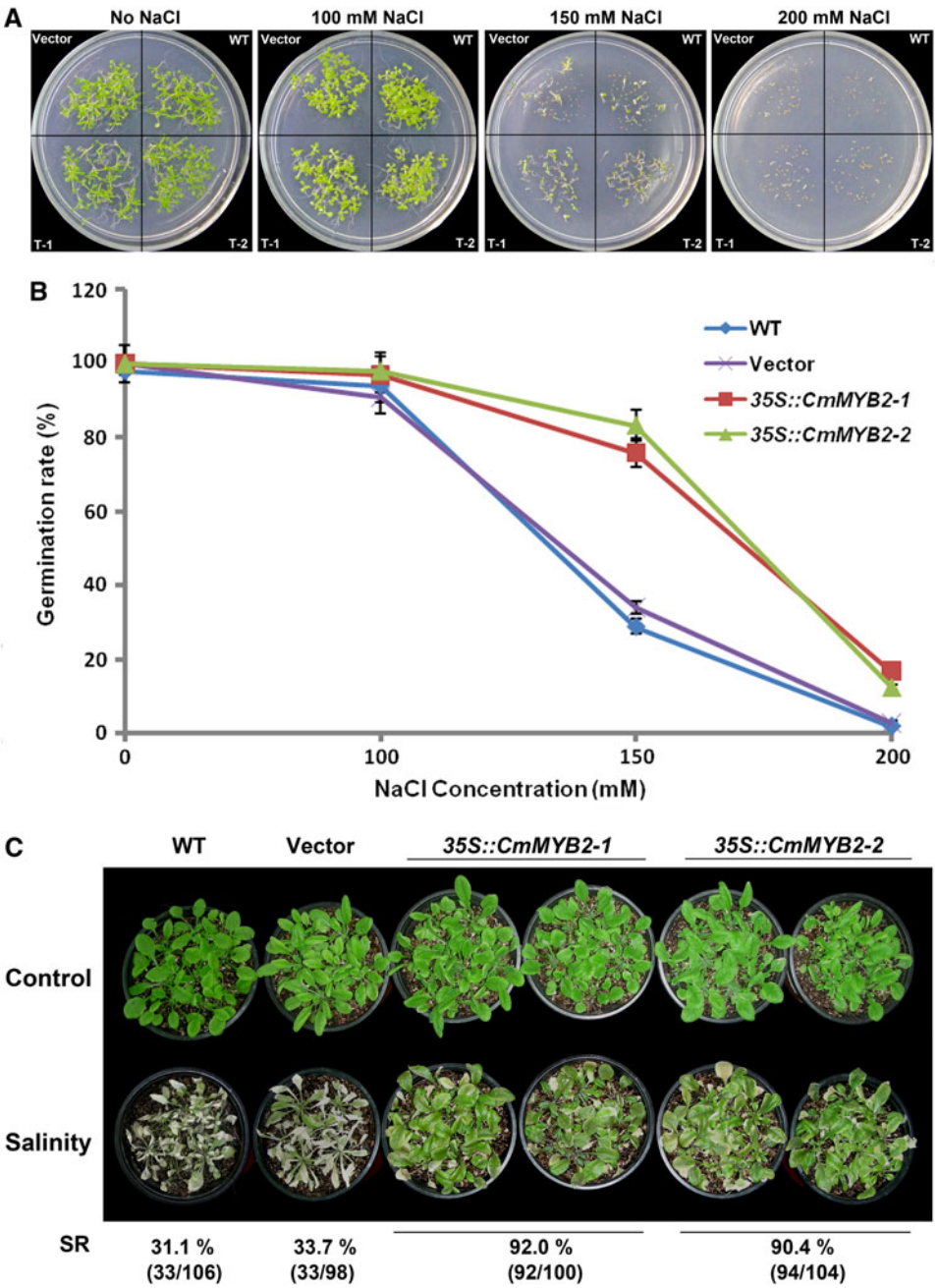


Fig. 5 Salinity tolerance in Col-0 and the *CmMYB2* transgenic *Arabidopsis* plants. **a** and **b** Germination of Col-0 and *CmMYB2* transgenic seeds in the presence of 0-, 100-, 150- and 200-mM NaCl. *T-1* and *T-2* indicate, respectively, *35S::CmMYB2-1* and *35S::CmMYB2-2*. Values represent the mean of three germination assays; **c** Salinity-challenged 3-week-old Col-0 and *CmMYB2* transgenic *Arabidopsis* plants irrigated with an increasing concentration of NaCl. *SR* survival rate ($n \geq 90$)

The four pathways interact with each other by several key regulatory genes, such as *CO*, *FLC*, *FT*, *SOC1*, *LFY* and *AP1* [34, 35]. *CO* is a floral enhancer and controls the timing of flowering in the photoperiod pathway by directly up-regulating *FT* and *SOC1* [36]. *FLC*, a strong floral repressor that antagonises the activity of vernalization and autonomous pathways, also acts as an upstream regulator of *FT* and *SOC1* [33]. The MADS-box flowering-time gene *SOC1* is regulated by several pathways and is proposed to co-ordinate responses to environmental signals and gibberellin signalling [37]. For the floral meristem identity genes, *AP1* is directly induced by *FT* [38], and *LFY* is both directly induced by *SOC1* and GA signal [39, 40]. In addition, as an important stress-related hormone, ABA is often antagonistic to GA; thus, it is not surprising that ABA has been shown to inhibit floral formation. Takeno and Maeda [41] found that for *Pharbitis nil* treated with exogenous ABA, the flower-inhibiting effect was observed.

In *Phalaenopsis hybrid*, ABA displayed an inhibiting response to flowering [42]. Signaling of ABA alters flowering through DELLA proteins which are essential for GA signaling has also been reported [43]. In this study, we found that the expression level of the *CO*, *FT*, *SOC1*, *LFY* and *AP1* was less in the *CmMYB2* transgenic *Arabidopsis* plants compared with Col-0 (Fig. 3), suggesting that the delayed flowering phenotype of *CmMYB2* transgenic *Arabidopsis* might be an integrated result of complex changes in the flowering regulatory network, correlated with the down-regulation by *CmMYB2* of some positive regulation genes in the flowering-time pathways.

CmMYB2 Enhanced Abiotic Tolerance by Inducing the Expression of Stress Responsive Genes

Heterologous expression in *A. thaliana* of *Craterostigma plantagineum MYB10* has been shown to increase ABA hypersensitivity and enhance both drought and salinity tolerance [44]. Similarly, over-expressors of *AtMYB2* showed enhanced tolerance to drought and salinity stress [14]. In the present study, the heterologous expression of *CmMYB2* improved the level of both drought and salinity tolerance in *A. thaliana* (Figs. 5 and 6). A number of studies have shown that ABA plays an important role in the stress response and tolerance of plants to drought and high salinity [1, 45]. ABA synthesis genes both *ABA1* and *ABA2* were up-regulated in the *CmMYB2* transgenics under salinity stress (Fig. 7f, g), raising the possibility that ABA is more effectively synthesized in these transgenic plants than in wild type ones, which might partially explain the enhanced abiotic stress tolerance in *CmMYB2* transgenic plants.

ABA is essential for plant normal growth and that in high concentrations, it has an inhibitory effect on seed germination and seedling growth [46]. Here, exogenous ABA inhibited the germination of the *CmMYB2* transgenic seed more severely than the wild type seed (Fig. 4a), suggesting that *CmMYB2* heightens ABA sensitivity during germination. Similar phenotypes have arisen in over-expressors of various ABA-dependent stress-responsive genes [47–50]. When treated with ABA, the stomatal apertures in the leaves of the *CmMYB2* transgenic plants were smaller than in Col-0 plants (Fig. 4b). Reduced stomatal aperture in response to ABA treatment has also been observed in over-expressors of *AtMYB61* [51] and *AtMYB15* [16]. Thus, we suggest that *CmMYB2* may function as a positive regulator of ABA-mediated stomatal closure. We found that expression of genes encoding a group of serine/threonine protein phosphatases 2C (PP2Cs), such as *ABI1*, *HAB1* and *HAB2*, was diminished in *CmMYB2* transgenics (Fig. 7h–j). These proteins belong to group A PP2Cs in *Arabidopsis* [52] and have been described as negative regulators of ABA signaling [53–56]. The recessive loss of function of *ABI1* mutants *abi1* was slightly more sensitive to ABA and showed a greater level of drought tolerance than wild type [53, 54]. The over-expression of *HAB1* led to impaired stomatal closure, promoted ABA-resistant root growth and diminished ABA-induced gene expression [52, 57]. In addition, a T-DNA knockout of *HAB1* conferred ABA hypersensitivity in the regulation of stomatal closure and germination [58]. As the putative paralog of *HAB1*, *HAB2* activity partially masked of the loss of function of *HAB1* [55]. Therefore, it is possible that ABA signalling is more effectively enhanced in the *CmMYB2* transgenics as a result of the partial

Fig. 7 The expression of stress-responsive genes *RD22*, *RD29A*, *RAB18*, *COR47*, *COR15*, *ABA1*, *ABA2*, *ABI1*, *HAB1*, *HAB2* (and *CmMYB2*) in Col-0 and the two *CmMYB2* transgenics. Values calculated from three replicates ▶

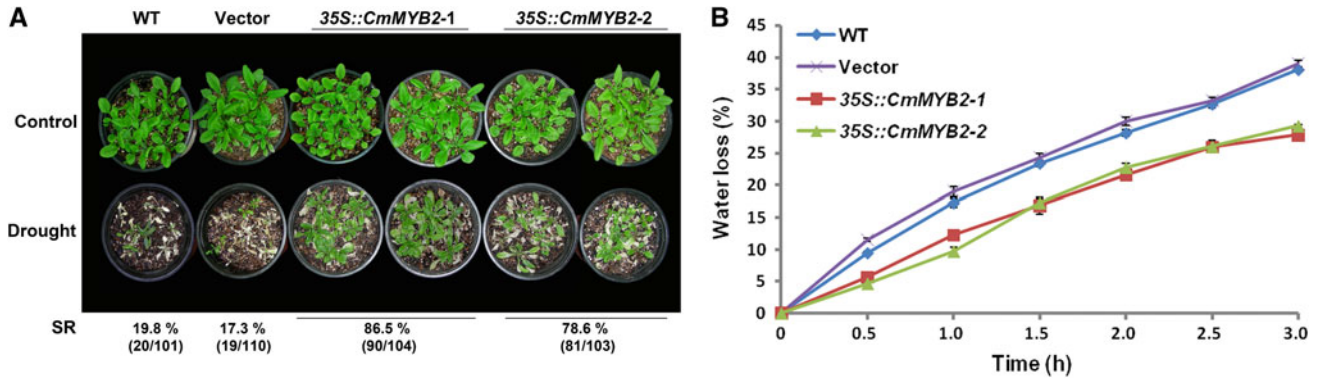
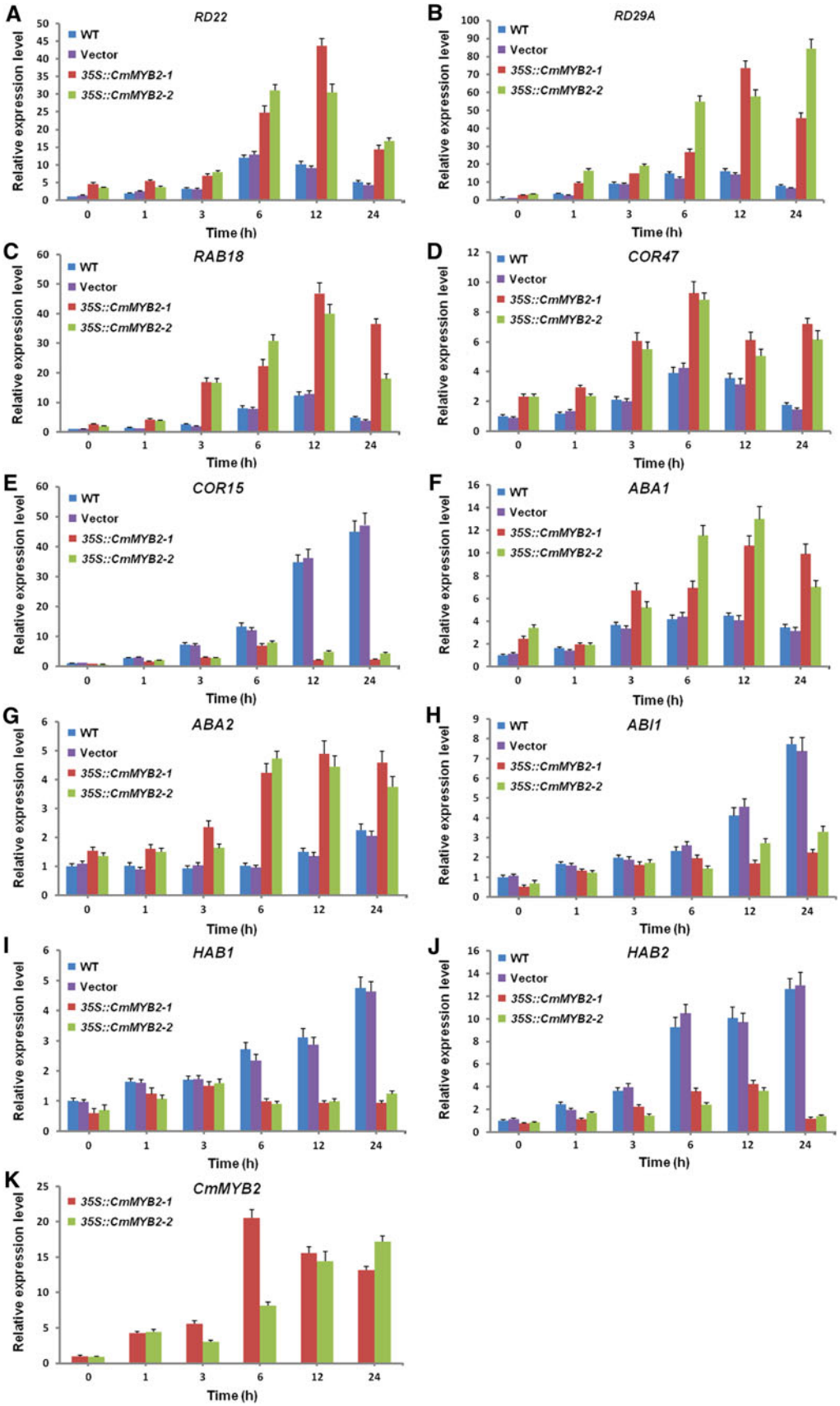


Fig. 6 Drought tolerance of Col-0 and *CmMYB2* transgenic *Arabidopsis* plants. **a** Two-week-old Col-0 and *CmMYB2* transgenic *Arabidopsis* plants were not watered for 14 days and then allowed to recover for 7 days. *SR* survival rate ($n \geq 100$); **b** Water loss in detached leaves. Mean values and standard deviation calculated from triplicated assays, with ten fully expanded rosette leaves used per genotype per assay


A
RD22
B
RD29A
C
RAB18
D
COR47
E
COR15
F
ABA1
G
ABA2
H
ABI1
I
HAB1
J
HAB2
K
CmMYB2
WT
Vector
35S::CmMYB2-1
35S::CmMYB2-2
Relative expression level
Time (h)

suppression of these PP2Cs genes, which is consistent with the results in *35S:AtMYB44* transgenic *Arabidopsis* plants under salt stress [18].

Increase in the expression level of *CmMYB2* was observed in *CmMYB2* transgenic *Arabidopsis* subjected to 250-mM NaCl (Fig. 7k). Chung et al. previously reported that salinity can stabilize the *SOS1* mRNA in *Arabidopsis* [59]. Because the 35S promoter is not an inducible promoter, we suppose that the increase in expression level of *CmMYB2* under salinity might result from stress-induced stability of the *CmMYB2* mRNA. However, additional evidence is needed before we can make such a conclusion. Moreover, the presence of the *CmMYB2* transgene altered the expression patterns of various stress-related genes. *RD22*, *RD29A*, *RAB18* and *COR47* are all up-regulated when *A. thaliana* is subjected to either salinity, drought or cold stress, or treated with exogenous ABA [60–66]. The increased expression of these stress response genes is thought to be advantageous for plants coping with abiotic stress [67, 68]. Here, the abiotic stress-associated genes *RD22*, *RD29A*, *RAB18* and *COR47* were all induced to a greater extent in the *CmMYB2* transgenics, especially in salinity-stressed plants (Fig. 7a–d).

The present data suggest that the heightened level of abiotic stress tolerance brought about by the constitutive expression of *CmMYB2* in *A. thaliana* may have come about through an increase in the rate of ABA synthesis and more effective ABA signalling. Together, these would act to deliver a higher level of expression of some key abiotic stress responsive genes. *CmMYB2* might be a promising gene for chrysanthemum drought and salinity tolerance improvement as well as flowering-time manipulation.

Acknowledgments This work was supported by the National Natural Science Foundation of China (Grant No. 30872064, 31071820, 31071825), and the Program for New Century Excellent Talents in University of Chinese Ministry of Education (Grant No. NCET-10-0492), and the Fundamental Research Funds for the Central Universities (KYJ 200907, KYZ201112), the Program for Hi-Tech Research, Jiangsu, China, Grant (No. BE2010303), 948 Project of Ministry of Agriculture (Grant No. 2008-G3), by Qing Lan Project of Jiangsu Province (Grant No. 2008 [30]), and the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

References

1. Nakashima, K., Ito, Y., & Yamaguchi-Shinozaki, K. (2009). Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. *Plant Physiology, 149*, 88–95.
2. Shinozaki, K., & Yamaguchi-Shinozaki, K. (2007). Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany, 58*, 221–227.
3. Schwechheimer, C., Zourelidou, M., & Bevan, M. W. (1998). Plant transcription factor studies. *Annual Review of Plant Physiology and Plant Molecular Biology, 49*, 127–150.
4. Singh, K., Foley, R. C., & Onate-Sa′nchez, L. (2002). Transcription factors in plant defense and stress responses. *Current Opinion in Plant Biology, 5*, 430–436.
5. Rosinsky, J. A., & Atchley, W. R. (1998). Molecular evolution of the Myb family of transcription factors: Evidence for polyphyletic origin. *Journal of Molecular Evolution, 46*, 74–83.
6. Jin, H., & Martin, C. (1999). Multifunctionality and diversity within the plant MYB-gene family. *Plant Molecular Biology, 41*, 577–585.
7. Stracke, R., Werber, M., & Weisshaar, B. (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. *Current Opinion in Plant Biology, 4*, 447–456.
8. Kranz, H. D., Denekamp, M., Greco, R., Jin, H., Leyva, A., Meissner, R. C., et al. (1998). Towards functional characterisation of the members of the R2R3-MYB gene family from *Arabidopsis thaliana*. *Plant Journal, 16*, 263–276.
9. Tamagnone, L., Merida, A., Parr, A., Mackay, S., Culianez-Macia, F. A., Roberts, K., et al. (1998). The AmMYB308 and AmMYB330 transcription factors from antirrhinum regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. *Plant Cell, 10*, 135–154.
10. Gocal, G. F. W., Sheldon, C. C., Gubler, F., Moritz, T., Bagnall, D. J., MacMillan, C. P., et al. (2001). *GAMYB-like* genes, flowering, and gibberellin signaling in *Arabidopsis*. *Plant Physiology, 127*, 1682–1693.
11. Liu, L., Du, H., Tang, X. F., Wu, Y. M., Huang, Y. B., & Tang, Y. X. (2008). The roles of MYB transcription factors on plant defense responses and its molecular mechanism. *Hereditas, 30*, 1265–1271.
12. Payne, T., Clement, J., Arnold, D., & Lloyd, A. (1999). Heterologous myb genes distinct from *GL1* enhance trichome production when overexpressed in *Nicotiana tabacum*. *Development, 126*, 671–682.
13. Schmitz, G., Tillmann, E., Carriero, F., Fiore, C., & Theres, K. (2002). The tomato *Blind* gene encodes a MYB transcription factor that controls the formation of lateral meristems. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 1064–1069.
14. Urao, T., Yamaguchi-Shinozaki, K., Urao, S., & Shinozaki, K. (1993). An Arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *Plant Cell, 5*, 1529–1539.
15. Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2003). Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell, 15*, 63–78.
16. Ding, Z. H., Li, S. M., An, X. L., Liu, X., Qin, H. J., & Wang, D. W. (2009). Transgenic expression of *MYB15* confers enhanced sensitivity to abscisic acid and improved drought tolerance in *Arabidopsis thaliana*. *Journal of Genetics and Genomics, 36*, 17–29.
17. Cominelli, E., Sala, T., Calvi, D., Gusmaroli, G., & Tonelli, C. (2008). Overexpression of the Arabidopsis *AtMYB41* gene alters cell expansion and leaf surface permeability. *Plant Journal, 53*, 53–64.
18. Jung, C. K., Seo, J. S., Han, S. W., Koo, Y. J., Kim, C. H., Song, S. I., et al. (2008). Overexpression of *AtMYB44* enhances stomatal closure to confer abiotic stress tolerance in transgenic Arabidopsis. *Plant Physiology, 146*, 623–635.
19. Cheong, Y. H., Chang, H. S., Gupta, R., Wang, X., Zhu, T., & Luan, S. (2002). Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. *Plant Physiology, 129*, 661–677.
20. Fowler, S., & Thomashow, M. F. (2002). *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell, 14*, 1675–1690.

21. Kamei, A., Seki, M., Umezawa, T., Ishida, J., Satou, M., Akiyama, K., et al. (2005). Analysis of gene expression profiles in Arabidopsis salt overly sensitive mutants sos2–1 and sos3–1. *Plant, Cell, and Environment, 28*, 1267–1275.
22. Kuno, N., Moller, S. G., Shinomura, T., Xu, X. M., Chua, N. H., & Furuya, M. (2003). The novel MYB protein EARLY-PHYTOCHROME-RESPONSIVE1 is a component of a slave circadian oscillator in Arabidopsis. *Plant Cell, 15*, 2476–2488.
23. Wang, Z. Y., & Tobin, E. M. (1998). Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1(CCA1) gene disrupts circadian rhythms and suppresses its own expression. *Cell, 93*, 1207–1217.
24. Chen, S. M., Miao, H. B., Chen, F. D., Jiang, B. B., Lu, J. G., & Fang, W. M. (2009). Analysis of expressed sequence tags (ESTs) collected from the inflorescence of Chrysanthemum. *Plant Molecular Biology Reporter, 27*, 503–510.
25. Murray, M. G., & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research, 8*, 4321–4326.
26. Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: Improving the sensibility of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research, 22*, 4673–4680.
27. Otsuga, D., DeGuzman, B., Prigge, M. J., Drews, G. N., & Clark, S. E. (2001). *REVOLUTA* regulates meristem initiation at lateral positions. *Plant Journal, 25*, 223–236.
28. Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-(Delta DeltaC(T)) method. *Methods, 25*, 402–408.
29. Clough, S. J., & Bent, A. F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal, 16*, 735–743.
30. Pei, Z. M., Kuchitsu, K., Ward, J. M., Schwarz, M., & Schroeder, J. I. (1997). Differential abscisic acid regulation of guard cell slow anion channels in *Arabidopsis* wild-type and *abi1* and *abi2* mutants. *Plant Cell, 9*, 409–423.
31. Hugouvieux, V., Kwak, J. M., & Schroeder, J. I. (2001). A mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in *Arabidopsis*. *Cell, 106*, 477–487.
32. Denekamp, M., & Smeekens, S. C. (2003). Integration of wounding and osmotic stress signals determines the expression of the *AtMYB102* transcription factor gene. *Plant Physiology, 132*, 1415–1423.
33. Putterill, J., Laurie, R., & Macknight, R. (2004). It's time to flower: The genetic control of flowering time. *Bioessays, 26*, 363–373.
34. Komeda, Y. (2004). Genetic regulation of time to flower in *Arabidopsis thaliana*. *Annual Review of Plant Biology, 55*, 521–535.
35. Boss, P. K., Bastow, R. M., Mylne, J. S., & Dean, C. (2004). Multiple pathways in the decision to flower: Enabling, promoting, and resetting. *Plant Cell, 16*(Suppl), S18–S31.
36. Hayama, R., & Coupland, G. (2003). Shedding light on the circadian clock and the photoperiodic control of flowering. *Current Opinion in Plant Biology, 6*, 13–19.
37. Hepworth, S. R., Valverde, F., Ravenscroft, D., Mouradov, A., & Coupland, G. (2002). Antagonistic regulation of flowering-time gene SOC1 by CONSTANS and FLC via separate promoter motifs. *EMBO Journal, 21*, 4327–4337.
38. Liljegren, S. J., Gustafson-Brown, C., Pinyopich, A., Ditta, G. S., & Yanofsky, M. F. (1999). Interactions among *APETALA*, *LEAFY*, and *TERMINAL FLOWER1* specify meristem fate. *Plant Cell, 11*, 1007–1018.
39. Lee, J., Oh, M., Park, H., & Lee, I. (2008). SOC1 translocated to the nucleus by interaction with AGL24 directly regulates *LEAFY*. *Plant Journal, 55*, 832–843.
40. Blázquez, M. A., Soowal, L. N., Lee, I., & Weigel, D. (1997). *LEAFY* expression and flower initiation in *Arabidopsis*. *Development, 124*, 3835–3844.
41. Takeno, K., & Maeda, T. (1996). Abscisic acid both promotes and inhibits photoperiodic flowering of *Pharbitis nil*. *Physiologia Plantarum, 98*, 467–470.
42. Wang, W. Y., Chen, W. S., Chen, W. H., Hung, L. S., & Chang, P. S. (2001). Influence of abscisic acid on flowering in *Phalaenopsis hybrid*. *Plant Physiology and Biochemistry, 40*, 97–100.
43. Achard, P., Cheng, H., Grauwe, L. D., Grauwe, L. D., Decat, J., Schoutteten, H., et al. (2006). Integration of plant responses to environmentally activated phytohormonal signals. *Science, 311*, 91–94.
44. Villalobos, M. A., Bartels, D., & Iturriaga, G. (2004). Stress tolerance and glucose insensitive phenotypes in Arabidopsis overexpressing the *CpMYB10* transcription factor gene. *Plant Physiology, 135*, 309–324.
45. Yamaguchi-Shinozaki, K., & Shinozaki, K. (2006). Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stress. *Annual Review of Plant Biology, 57*, 781–803.
46. Finkelstein, R. R., Gampala, S. S. L., & Rock, C. D. (2002). Abscisic acid signaling in seeds and seedlings. *Plant Cell, 14*, S15–S45.
47. Sakuma, Y., Maruyama, K., Osakabe, Y., Qin, F., Seki, M., Shinozaki, K., et al. (2006). Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. *Plant Cell, 18*, 1292–1309.
48. Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., et al. (2006). Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant and Cell Physiology, 47*, 141–153.
49. Hu, H. H., You, J., Fang, Y. J., Zhu, X. Y., Qi, Z. Y., & Xiong, L. Z. (2008). Characterization of transcription factor gene *SNAC2* conferring cold and salt tolerance in rice. *Plant Molecular Biology, 67*, 169–181.
50. Kang, J. Y., Choi, H. I., Im, M. Y., & Kim, S. Y. (2002). Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. *Plant Cell, 14*, 343–357.
51. Liang, Y. K., Dubos, C., Dodd, I. C., Holroyd, G. H., Hetherington, A. M., & Campbell, M. M. (2005). *AtMYB61*, an R2R3-MYB transcription factor controlling stomatal aperture in *Arabidopsis thaliana*. *Current Biology, 15*, 1201–1206.
52. Schweighofer, A., Hirt, H., & Meskiene, I. (2004). Plant PP2C phosphatases: Emerging functions in stress signaling. *Trends in Plant Science, 9*, 236–243.
53. Gosti, F., Beaudoin, N., Serizet, C., Webb, A. A. R., Vartanian, N., & Giraudat, J. (1999). ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. *Plant Cell, 11*, 1897–1910.
54. Merlot, S., Gosti, F., Guerrier, D., Vavasseur, A., & Giraudat, J. (2001). The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid pathway. *Plant Journal, 25*, 295–303.
55. Sáez, A., Apostolova, N., González-Guzmán, M., González-García, M. P., Nicolás, C., Lorenzo, O., et al. (2004). Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C *HAB1* reveal its role as a negative regulator of abscisic acid signalling. *Plant Journal, 37*, 354–369.
56. Kuhn, J. M., Boisson-Dernier, A., Dizon, M. B., Maktabi, M. H., & Schroeder, J. I. (2006). The protein phosphatase *AtPP2CA* negatively regulates abscisic acid signal transduction in Arabidopsis, and effects of *abh1* on *AtPP2CA* mRNA. *Plant Physiology, 140*, 127–139.
57. Sáez, A., Robert, N., Maktabi, M. H., Schroeder, J. I., Serrano, R., & Rodriguez, P. L. (2006). Enhancement of abscisic acid

sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatase type 2C ABI1 and HAB1. *Plant Physiology, 141*, 1389–1399.
58. Leonhardt, N., Kwak, J. M., Robert, N., Waner, D., Leonhardt, G., & Schroeder, J. I. (2004). Microarray expression analyses of *Arabidopsis* guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. *Plant Cell, 16*, 596–615.
59. Chung, J. S., Zhu, J. K., Bressan, R. A., Hasegawa, P. M., & Shi, H. Z. (2008). Reactive oxygen species mediate Na^+-induced *SOS1*mRNA stability in Arabidopsis. *Plant Journal, 53*, 554–565.
60. Lång, V., & Palva, E. T. (1992). The expression of a *rab*-related gene, *rab18*, is induced by abscisic acid during the cold acclimation process of *Arabidopsis thaliana* (L.) Heynh. Plant. *Molecular Biology, 20*, 951–962.
61. Albinsky, D., Masson, J. E., Bogucki, A., Afsar, K., Vass, I., Nagy, F., et al. (1999). Plant responses to genotoxic stress are linked to an ABA/salinity signaling pathway. *Plant Journal, 17*, 73–82.
62. Close, T. J. (1997). Dehydrins: a commonalty in the response of plants to dehydration and low temperature. *Physiologia Plantarum, 100*, 291–296.
63. Nylander, M., Svensson, J., Palva, E. T., & Welin, B. V. (2001). Stress-induced accumulation and tissue-specific localization of dehydrins in *Arabidopsis thaliana*. *Plant Molecular Biology, 45*, 263–279.
64. Shinozaki, K., & Yamaguchi-Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: Differences and cross-talk between two stress signaling pathways. *Current Opinion in Plant Biology, 3*, 217–223.
65. Yamaguchi-Shinozaki, K., & Shinozaki, K. (1993). Characterization of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. *Molecular and General Genetics, 236*, 331–340.
66. Yamaguchi-Shinozaki, K., & Shinozaki, K. (1993). The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression of *rd22*, a gene responsive to dehydration stress in *Arabidopsis thaliana*. *Molecular and General Genetics, 238*, 17–25.
67. Shinozaki, K., & Yamaguchi-Shinozaki, K. (1997). Gene expression and signal transduction in water-stress response. *Plant Physiology, 115*, 327–334.
68. Zhu, J. K. (2002). Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology, 53*, 247–273.