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CaMKK1 from *Chenopodium album* positively regulates salt and drought tolerance in transgenic tobacco

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Abstract Plants encounter various abiotic stresses during the whole life cycle. The mitogen-activated protein kinase kinase (MAPKK) acts as the convergent point of MAPK cascade in stress signaling pathway, which has not widely been studied so far. In the present study, we isolated a group A *MAPKK* gene (named as *CaMKK1*) from *Chenopodium album*, a salt-tolerant plant species in Chenopodiaceae. Quantitative RT-PCR analysis indicates that *CaMKK1* was significantly up-regulated and displayed a fast and transient activation, which was similar with MAPK expression pattern in *C. album* under salt and drought stress. Ectopic expression of *CaMKK1* in *Escherichia coli* BL21 (DE3) strains showed that overexpression of *CaMKK1* could increase the salt and drought tolerance to bacterium. Overexpression of *CaMKK1* in transgenic tobacco exhibited attenuated salt and PEG sensitivity by means of improving germination percentage and seedling growth. Furthermore, transgenic tobacco lines significantly accumulated organic osmoprotectants, and increased antioxidant enzyme activities which effectively relieved the accumulated reactive oxygen species under stress; the water loss rate was reduced and net photosynthetic rate was significantly increased in transgenic lines compared to non-transgenic plant. The more worth mentioning is that the transcripts of downstream transcription factors (*NtDREB2, NtDREB3, NtDREB4*) were quickly and dramatically accumulated (from 15 to 224 folds) in transgenic lines under stress. In conclusion, our results indicate that *CaMKK1* is a positive regulator in response to salt and drought stress in plants, which should provide new data for further analyzing the function of plant MAPK pathway.

Keywords Abiotic stress · *Chenopodium album* ·
CaMKK1 · ROS · Transgenic tobacco lines

Introduction

Plants constantly encounter different stresses in growth and developmental process because of sessility (Long et al. 2013). To cope with, they have developed a variety of mechanisms, including morphological, physiological and molecular aspects. Out of these, signal transduction pathway is the particularly important strategy for plant adaptation to harsh circumstances (Shinozaki et al. 2003; Huang et al. 2012). Mitogen-activated protein kinase (MAPK) cascade is one of the well-known signaling pathways of plant in response to adversity (Smekalova et al. 2014), which comprises three functionally linked protein kinases, MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. Following successive phosphorylation, signals can be transferred from upstream to a variety of downstream components (Hirt 1997; MAPK group 2002; Rodriguez et al. 2010). Plant MAPKKs are present with the least members in the MAPK signaling modules (Takahashi

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et al. 2009), e.g. in Arabidopsis, only ten MAPKK genes were identified compared to 20 MAPK and more than 60 MAPKKK. So far, a variety of MAPKKs have been characterized from poplar (Hamel et al. 2006), rice (Singh et al. 2012), maize (Kong et al. 2013), etc. Generally, signals perceived by MAPKKKs are integrated and transduced by MAPKKs, and the specificity of cellular response regulated by MAPK module is mainly determined by MAPKK, suggesting that MAPKKs play critical role in transforming extracellular stimuli into cellular responses in MAPK cascade (Andreasson and Ellis 2010). Transgenic plants with *MAPKK* overexpression provide further evidence in plant response to cold, salt, drought, heat, etc. stresses (Agarwal et al. 2010; Kiegerl 2000; Liu et al. 2015; Mikołajczyk et al. 2000; Mizoguchi et al. 1998; Wu et al. 2015). Site-direction mutagenesis of *OsMKK6* from rice (*Oryza sativa*) makes the salt tolerance function of this gene constitutively active (Kumar and Sinha 2013). Not all members of MAP-KKs respond to stress positively, some of them apply opposite effect. Overexpression of *GhMKK1* in cotton enhances while *GhMKK5* reduces salt and drought tolerance (Lu et al. 2013; Zhang et al. 2012). Analysis of *BdMKK6.2* (*Brachypodium distachyon*) reveals a negative regulation of drought tolerance by influencing reactive oxygen species (ROS) homeostasis and transcription of stress-responsive genes (Sun et al. 2016).

It has been reported that a cross-talk between MAPK cascade and ROS signaling is involved in plant response to various abiotic stresses, e.g. MEKK1-MKK1/MKK2-MPK4 cascade in ROS homeostasis in *A. thaliana*, because some redox-related genes failed to express in *mekk1* or *mpk4* mutants (Nakagami et al. 2006). Previous studies indicated that *AtMKK1* induced *CAT1* (catalase) expression by triggering H_2O_2 production in response to drought and salt stress (Xing et al. 2007). In addition, several transcription factors which act downstream of MAPK cascade in ROS response have been identified, e.g. transcription factor AtMYB44 (Persak and Pitzschke 2014), NbWRKY8 (Ishihama et al. 2011), etc. However, currently other downstream transcription factor, e.g. DREB, etc. in interaction with MAPK pathway components has not been well-documented.

Chenopodium album is a salt tolerant species (Hamidov et al. 2007) and widely distributes in the semi-arid area in Xinjiang province, China (Commissione Redactorum Florae Xinjiangensis 1994). It shows very strong adaptability to harsh environments, however, no specialized structures, e.g. assimilation shoot, succulent or degenerated leaf, etc. are employed by *C. album* in dealing with the heterogeneous circumstances. We speculate that it must have some special way (e.g. physiological or molecular mechanism) in adaption of the unpredictable conditions, so, in the present study, we cloned the coding sequence of *MAPKK* from *C. album*, and characterized its responses to salt and drought stress in transgenic tobacco. In comparison with non-transgenic plants, we found that overexpression of *CaMKK1* reduced the sensitivity to salt and drought in seed germination, seedling and adult plant growth, which corresponded to changes of various physiological parameters, e.g. organic osmoprotectant contents, anti-oxidant enzyme activities, water loss rate, net photosynthesis rate, etc. More interestingly, *CaMKK1* overexpression dramatically increased the expression level of three transcription factors-*DREB2, DREB3, DREB4* (dehydration response element binding factors, DREB), which is the first report on the quick and remarkable response of downstream DREB transcription factors to MAPKK. Our data suggest that *CaMKK1* is a positive regulator which promotes the expression of downstream transcription factor in MAPK cascade and confers stress tolerance to transgenic tobacco.

Materials and methods

Plant cultivation and treatments

Seeds of *C. album* were sown in pots containing a 3:1 mixture of vermiculite:perlite (v/v) in the growth chamber under a 16 h light/8 h dark photoperiod, 25 °C, 10–20% relative humidity. Plants were cultivated for 4–6 weeks [well-watered and with Hoagland solution supplying at an interval of 2–3 weeks] before next use.

For quantitative RT-PCR (qPCR) analysis of *CaMKK1*, the above plants were treated with Hoagland solution containing 50, 100, 300 mM NaCl (for NaCl stress), or 0, 5, 10, 15% polyethylene glycol (PEG) 6000 (for mimic drought stress), then sampling at 0, 0.5, 1, 2, 5, 12, 24, 48 h time points during treatment, using Hoagland solution only as control. For qRT-PCR analysis of *CaMPK*, 300 mM NaCl or 20% PEG was applied for stress treatment. For qPCR analysis of stress-related genes in transgenic tobacco lines, seeds of non-transgenic (NT) and transgenic (T1) tobacco lines were surface-sterilized and sown on MS medium only (for NT) and MS containing 250 mg L^{-1} Kanamycin (for T1), seedlings grew till two leaves (1–2 cm in diameter) were present and NT seedlings would become yellowish. The green seedlings were transferred into fresh MS medium for 1 week and subjected to PCR identification, the positive seedlings grew for another 1 month till four leaves were present and the height reached to 10 cm, then transferred into vermiculite:perlite (3:1, v/v) mixture and managed as mentioned above. For assays, four samples were collected from young fresh leaves in the upper part of the plants, and immediately frozen in liquid nitrogen until use.

Total RNA isolation

Young fresh leaves (0.12 g) were harvested and total RNA was extracted by using the HP Plant RNA Kit (R6837-02, Omega, US). Each reverse transcription reaction was performed with total RNA (1.5 μg) by using the M-MLV reverse transcriptase kit (TaKaRa, Dalian, China) according to the manufacturer's instructions.

Cloning of *CaMKK1* and construction of prokaryotic and plant expression vectors

The cDNA fragment of *MAPKK* open reading frame (ORF) from *C. album* was amplified using the above cDNA template with the primers shown in Table 1. The PCR product was cloned into the pMD-18-T vector (TaKaRa, Dalian, China) and sent for sequencing. The *CaMAPKK*cDNA with correct sequence (named as *CaMKK1*) was submitted to GenBank and received an accession number JQ994336. Consequently it was recombined into the prokaryotic expression vector as pET-28a-*CaMKK1* and plant expression vector pCAMBIA2300 under the control of the CaMV 35S promoter (shortened as *35S::CaMKK1*).

Generation of the transgenic tobacco lines

Germfree tobacco seedlings (5–6 week-old) were transformed with *Agrobacterium* strain EHA105 in which *35S::CaMKK1* was harboring by using tobacco leaf disc transformation method (Horsch et al. 1985). Regeneration tobacco plantlets with 2–3 leaves [non-transgenic plant (NT) or *CaMKK1* transformed] were transplanted in soil and cultivated in greenhouse with a 16 h light/8 h dark photoperiod, 25 °C, 20% relative humidity. The seeds from

regenerated plants of PCR positive (T0) were harvested and screened on MS medium containing 250 mg L^{-1} kanamycin for 10–15 days. Kanamycin-resistant seedlings were identified by PCR and RT-PCR to confirm the gene integration in genome (T1). Three representative *CaMKK1*-overexpressed lines (OE1, OE2, OE3) of T1 generation tobacco were selected for further functional assays. For analysis of seed germination and seedling growth, the transgenic seeds of T2 generation were harvested from the three lines of T1 generation and applied in the measurement. For PCR identification, genomic DNA from transgenic plant was extracted according to instruction of Tiangen Plant Genomic DNA Extraction Kit (Tiangen, Beijing, China) by using primers described in Table 1 [*CaMKK1* (ORF)], PCR was performed in the following conditions: 94 °C 5 min; 35 cycles at 94 °C 30 s, 56 °C 30 s and 72 °C 1 min, followed by a final incubation at 72 °C for 10 min.

Analysis of salt and drought tolerance with *CaMKK1* overexpression

Effect of recombinant CaMKK1 on prokaryotic cell growth E. coli

BL21 (DE3) strains with recombinant plasmid (pET-28a-*CaMKK1*) and control vector (pET-28a) were inoculated in LB liquid medium containing 100 mg L^{-1} kanamycin and grown at 37 °C overnight. The cultures were then re-inoculated to fresh LB medium containing 100 mg L^{-1} kanamycin (1% culture in 20 mL LB in a 100 mL conical flask), which continued to grow for 4 h (about 0.5 OD$_{600}$), then were induced with 0.5 mM IPTG to express recombinant protein for another 4 h. Each culture (200 μL of 0.8 OD$_{600}$) was inoculated into 20 mL fresh LB medium (with

Table 1 Primers used in gene cloning and qPCR analysis in the present study

Gene	Primer sequence 5′-3′	
	Forward	Reverse
CaMKK1 (ORF)	GC<u>GAGCTC</u>ATGCCCAACAGAG*	GG<u>TCGAC</u>CCTACAAGGTTGTGAGTG*
CaMKK1 (qPCR)	CAGAAAGATCCAAAGGCTAGGC	CAGAAAGATCCAAAGGCTAGGC
CaMPK (qPCR)	CCTCCTCTTCGTCCTATCGGTAGA	AAGTTCTCTCTCTGCGGTGGTTT
NtMPK 4 (qPCR)	ACATCCGAGACAGATTTCAT	GTCTTGTCATCATCTCACCC
NtDREB2 (qPCR)	GGGTCGTTCTGCTTGCTTGA	GAGTAGTGCCTGAATCTGCTTCTTC
NtDREB3 (qPCR)	CAGGCAAGTGGGTTTCTGAAGTC	GAGGAAGCAGGAACAGGCAACT
NtDREB4 (qPCR)	AATCTGGCTGGGCACTTTCC	CCACCACTAAGGACGATGTTTCC
NtSOD (qPCR)	TGCAGCTCCACCACCAGAAGCATCATCAGAC	GGCTCACCACCACCCTCGCGGACA
NtADC2 (qPCR)	GCTGATTACCGCAATTTATCTGCTGC	GATAATATTCCCCTTACTGCAGGC
NtLEA5 (qPCR)	CCAAACTCATCTCTGCTTTCG	AGCATGACTCTCTGGCCTGT
Caactin (qPCR)	AGCAACTGGGATGACATGGAGAAGATTTG	ACACCATCACCAGAATCCAGCACAATACC
Ntactin (qPCR)	TGGCTCAGAGAGGTTCAGATGTC	CCACCACTAAGGACGATGTTTCC

*Each of them contains *Sac* I or *Sal* I restriction endonuclease site (<u>underline</u>), respectively

100 mg L^{-1} kanamycin) containing 0, 50, 100, 200 mM NaCl, or 0, 5, 10, 15% PEG 6000, and incubated at 37 °C. Cultures (1.0 mL) were removed at an interval of 1 h for a total of 6 h and the optical density value at 600 nm (OD_{600}) was recorded by spectrophotometer (Benchmark Plus microplate, Bio-Rad, USA). Three replicates were applied with each treatment.

Effect of CaMKK1 overexpression in transgenic tobacco lines

To characterize salt or drought-stress sensitivity, we performed a germination and seedling growth assay. A total of 30 surface-sterilized seeds from homozygous T2 transgenic lines and non-transgenic (NT) tobacco plants were germinated on MS medium containing different concentrations of NaCl (0, 100, 200, 300 mM) or PEG 6000 (0, 5, 10, 15%) at 25 °C, with a 16 h light/8 h dark photoperiod. Each experiment was repeated at least three times with similar results. Germination percentage was recorded every 24 h during a 2-week period. The seedling growth was measured by calculating ten seedlings of the root length at the end of 2-week germination.

Measurement of physiological responses of *CaMKK1* overexpressed tobacco lines to abiotic stresses

Three-month-old tobacco transgenic lines (OE1, OE2, OE3; T1 generation) and NT plants were treated with 15% PEG 6000 or 300 mM NaCl for 48 h, respectively. For assay of the organic osmoprotectants-proline, soluble sugar (SS) or glycine betaine (GB), young fresh leaves (0.15–0.2 g) were used in extraction. Proline concentration was determined by using aqueous sulfosalicylic acid to extract and acidic ninhydrin to develop the color according to Zhao et al. method (Zhao et al. 2009); GB was determined by using Lokhande et al. method (Lokhande et al. 2010); the concentration of SS was determined by using anthrone-sulphuric acid colorimetric method described by Palma et al. (2009). For measuring O_2^- production rate, H_2O_2 concentration or the amount of malondialdehyde (MDA), young fresh leaves (0.15–0.2 g) were homogenized with different extraction buffers. O_2^- level was examined by using hydroxylamine oxidization method, H_2O_2 concentration was determined by titanium sulfate colorimetric method and the amount of MDA was determined by the thiobarbituric acid reaction according to Weydert and Cullen (2010). For measurement of activity of superoxide dismutase (SOD), peroxidase (POD) or catalase (CAT), young fresh leaves (0.15 g) were homogenized in ice-cold PBS buffer, after being filtered and centrifuged, the supernatant of the homogenate was used as crude enzyme immediately for analysis. SOD activity was determined by measuring its

ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT), as described by Basu et al. (2009); POD or CAT activity was defined as guaiacol increase at 470 nm or H_2O_2 decrease at 240 nm, according to the method of Yu et al. (2015).

Water loss experiment

Young fresh leaves of tobacco transgenic lines-OE1, OE2, OE3 and NT plants (3-month-old) were detached and placed on filter paper at room temperature, the fresh weight of each detached leaf was determined at 0, 10, 30, 60, 90, 120, 150, 180, 210 and 240 min, respectively, then the leaves were photographed when the dehydration was completed. The experiment was repeated at least for three times with the similar results.

Detection of H_2O_2 and superoxide anion accumulation by 3,3′-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) staining

Non-transgenic plants and transgenic lines were subjected to 200 mM NaCl or 15% PEG 6000 treatment for 2 h before test. For H_2O_2 detection, young fresh leaves were incubated in DAB aqueous solution (1 mg mL^{-1}, pH 3.2) for overnight at 25 °C in the dark according to Kumar et al. (2013) method; superoxide anion was detected via NBT staining based on Kumar et al. (2013) method. In NBT assay, young fresh leaves were incubated in 0.2% NBT solution [0.1 g NBT in 50 mL 50 mM sodium phosphate buffer (pH 7.5)] for 24 h at 25 °C in the dark. Thereafter, the leaves (from the above two methods) were soaked in 95% ethanol and heated in a boiling water-bath for 10 min (or more if necessary, with intermittent shaking) to remove chlorophyll. At least three leaves from each treatment were stained in the experiment. The staining result was determined by doing statistical analysis with MATLAB software (vision 7.0) based on graphical user interface.

Measurement of the net photosynthetic rate and intercellular CO_2 concentration in transgenic tobacco

The net photosynthetic rate (Anet) and intercellular CO_2 concentration (Ci) of NT and three T1 transgenic lines (OE1, OE2, OE3) were measured before and after NaCl or PEG treatment for 2 h, 24 and 48 h by using a portable infrared gas analyzer-based photosynthesis system (Li-Cor 6400; Li-Cor, Lincoln, NE, USA) according to the method described by Zhang et al. (2014). For measurement, the third fully expanded leaf from the top of a plant with the least area of 5×5 cm^2 was taken into use. At least

ten repeat readings of each leaf and three leaves from three plants of each line were recorded.

Quantitative RT-PCR analysis of *CaMKK1* and *CaMPK* in *C. album* and the stress-related genes in *CaMKK1*-overexpressed transgenic tobacco lines under abiotic stresses

Total RNA was isolated from the young fresh leaves of *C. album* or tobacco (NT and transgenic lines) under different stresses, and the cDNA was synthesized according to the above procedure. Quantitative RT-PCR was carried out by using the ABI 7500 Real time PCR system (Applied Biosystem, US). Detection of qRT-PCR product was performed by staining with QuantiNova SYBR Green PCR Kit (Cat. 208054; Qiagen, Germany). For expression pattern of *CaMKK1* and *CaMPK* in *C. album*, the specific primers were shown in Table 1. The relative amplification of *β-actin* of *C. album* was used for normalization. For expression profile of the stress-related genes in tobacco transgenic lines, *NtMPK4*, three dehydration response element binding factor (DREB) genes (*NtDREB2, NtDREB3, NtDREB4*), stress response genes-group five late embryogenesis abundant protein (*NtLEA5*), arginine decarboxylase (*NtADC2*) and superoxide dismutase (*NtSOD*) were analyzed in the present study. The primer sequences were shown in Table 1. The relative amplification of *β-actin* of tobacco was used for normalization. The above amplification was performed in the following conditions: 95 °C 2 min followed by 40 cycles of 95 °C 5 s, 60 °C 30 s. Four samples (biological replicates) of each treatment were duplicated (technical replicates) in qRT-PCR experiment. The relative expression level of genes was quantified according to the $R = 2^{-\Delta\Delta C_T}$ mathematical model (Shi and Chiang 2005), where $\Delta\Delta C_T = \Delta C_{T \text{ target sample}} - \Delta C_{T \text{ control sample}}$, $\Delta C_{T \text{ sample}} = C_{T \text{ test gene}} - C_{T \text{ reference gene}}$. The final value of relative quantification was described as fold change of gene expression in the test sample compared to control.

Statistical analysis

All data were expressed as mean ± standard error. For seed germination, percentage was arcsine transformed from four replicates before statistical analysis; for root length, ten seedlings were measured; for physiological parameter assay, four replicates were measured; for qRT-PCR, four biological replicates and two technical replicates of each treatment were applied. Microsoft Excel and the software of GraphPad Prism 5.0 were employed in data analysis. One-way ANOVA Post Hoc Duncan's multiple range test was used in multiple variables comparison. Any significant difference compared to control at $P < 0.05$ or $P < 0.01$ was given.
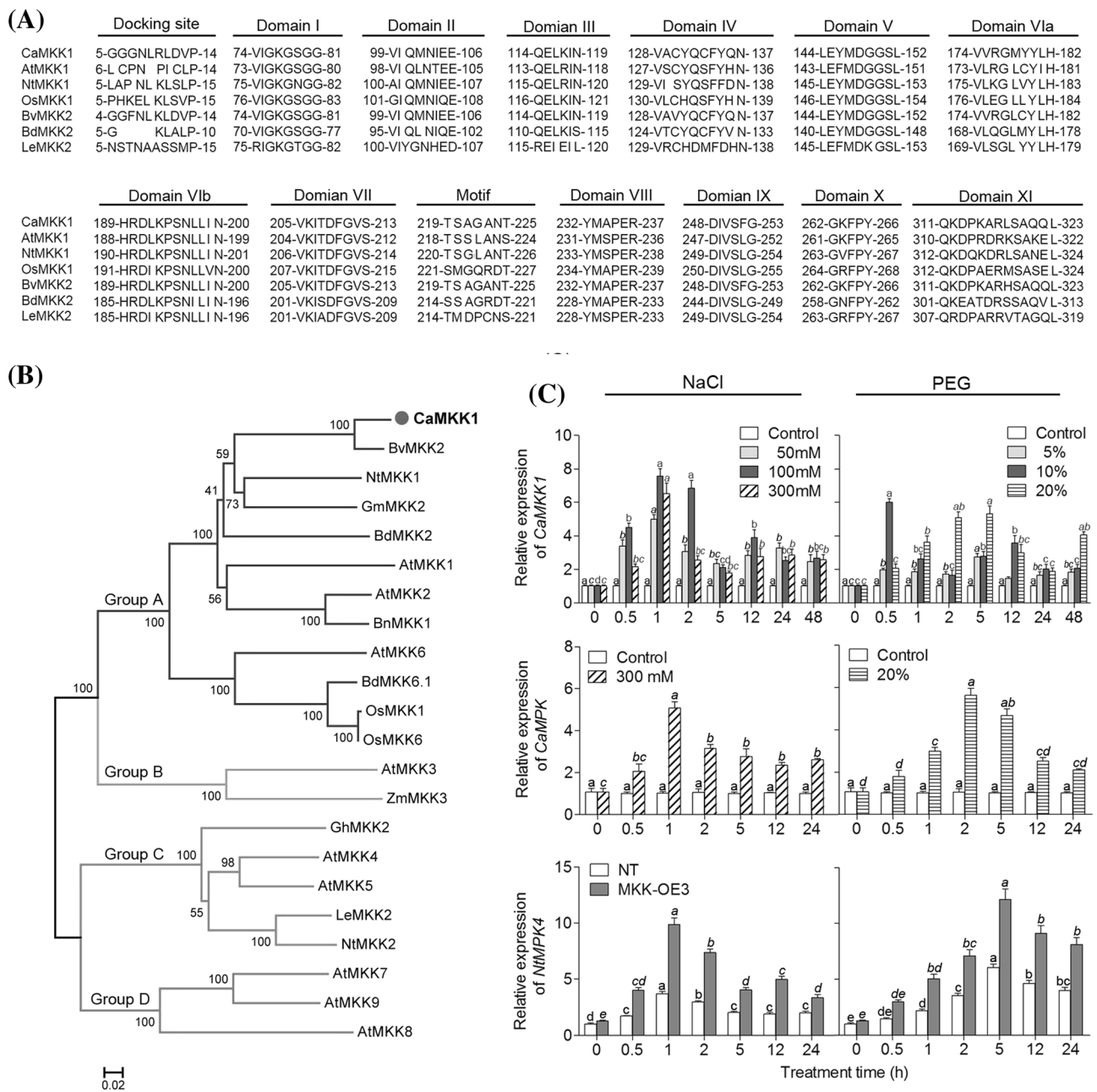
Results

Cloning and characterization of *CaMKK1* in *C. album*

A cDNA fragment of *CaMKK1* open reading frame (ORF) was obtained from *C. album*, which is 1062 bp long and encodes 353 putative amino acids with an estimated molecular weight of 36 kDa. Alignment of the deduced amino acid sequence of *CaMKK1* with MAPKKs from other plant species indicates that *CaMKK1* possesses all 11 conserved subdomains (I–XI) and contains a conserved S/TXXXXXS/T motif as the phosphorylation site between VII and VIII domains, and a docking site known as binding MAPKs or phosphatases (Fig. 1a).

To reveal the evolutionary relationship among MAPKKs from various plant species, we constructed a phylogenetic tree using amino acid sequences derived from the GenBank database (Fig. 1b). Blastp analysis showed that CaMKK1 shared a high degree of sequence similarity with BvMKK2 (XP_010696552.1) from *Beta vulgaris* subsp. *Vulgaris*, and which is a member of group A MAPKK.

To investigate the expression pattern of *CaMKK1* under abiotic stresses, we examined the transcript accumulation of *CaMKK1* in *C. album* by qRT-PCR. As shown in Fig. 1c (top), both NaCl and PEG treatments could induce expression of *CaMKK1*. For NaCl, the transcripts accumulated significantly from 50 to 300 mM concentration and reached to the highest level at a short time (1 h); PEG treatment revealed a little different expression pattern, in which a sharp increase of *CaMKK1* transcription at 10% PEG was observed at 0.5 h, while that of the other concentrations reached to the highest level at 5 h. These results suggest that *CaMKK1* can respond actively to abiotic stress.

To further explore the expression pattern of downstream component of *CaMKK1*, we analyzed the transcriptional level of *CaMPK* under 300 mM NaCl or 20% PEG treatment. The responses of *CaMPK* to salt and drought showed similar trends with those of *CaMKK1*, in which *CaMPK* was quickly induced under salt stress, and the transcripts were accumulated significantly to the highest level at 1 h (Fig. 1c, middle); upon exposure to 20% PEG, the expression level of *CaMPK* gradually increased and reached to the maximum at 2 h, which also showed a quick induction pattern. Meanwhile, we also analyzed the expression of *NtMPK4* in *CaMKK*-overexpressed transgenic tobacco line (OE3), which is a homolog of *CaMPK* in *N. tabacum* and can positively respond to stress (Zhang et al. 2013). Results revealed that the *NtMPK4* was quickly up-regulated by NaCl or PEG treatment, and the increment was significantly higher than that of the NT plant under stress (Fig. 1c, bottom). All these results suggest that *CaMKK1, CaMPK* and *NtMPK4* all respond to stress fast and transiently, and the former one can positively activate the downstream

(A)

	Docking site	Domain I	Domain II	Domian III	Domain IV	Domain V	Domain VIa
CaMKK1	5-GGGNLRLDVP-14	74-VIGKGSGG-81	99-VI QMNIEE-106	114-QELKIN-119	128-VACYQCFYQN-137	144-LEYMDGGSL-152	174-VVRGMYYLH-182
AtMKK1	6-L CPN PI CLP-14	73-VIGKGSGG-80	98-VI QLNTEE-105	113-QELRIN-118	127-VSCYQSFYHN-136	143-LEFMDGGSL-151	173-VLRG LCYI H-181
NtMKK1	5-LAP NL KLSLP-15	75-VIGKGNGG-82	100-AI QMNIEE-107	115-QELRIN-120	129-VI SYQSFFDN-138	145-LEYMDGGSL-153	175-VLKG LVY LH-183
OsMKK1	5-PHKEL KLSVP-15	76-VIGKGSGG-83	101-GI QMNIQE-108	116-QELKIN-121	130-VLCHQSFYH N-139	146-LEYMDGGSL-154	176-VLEG LL YLH-184
BvMKK2	4-GGFNL KLDVP-14	74-VIGKGSGG-81	99-VI QMNIEE-106	114-QELRIN-119	128-VAVYQCFYQN-137	144-LEYMDGGSL-152	174-VVRGLCY LH-182
BdMKK2	5-G KLALP-10	70-VIGKGSGG-77	95-VI QL NIQE-102	110-QELKIS-115	124-VTCYQCFYV N-133	140-LEYMDGGSL-148	168-VLQGLMY LH-178
LeMKK2	5-NSTNAASSMP-15	75-RIGKGTGG-82	100-VIYGNHED-107	115-REI EI L-120	129-VRCHDMFDHN-138	145-LEFMDK GSL-153	169-VLSGL YY LH-179

	Domain VIb	Domian VII	Motif	Domain VIII	Domian IX	Domain X	Domain XI
CaMKK1	189-HRDLKPSNLLI N-200	205-VKITDFGVS-213	219-T SAG ANT-225	232-YMAPER-237	248-DIVSFG-253	262-GKFPY-266	311-QKDPKARLSAQQ L-323
AtMKK1	188-HRDLKPSNLLI N-199	204-VKITDFGVS-212	218-T SS LANS-224	231-YMSPER-236	247-DIVSLG-252	261-GKFPY-265	310-QKDPRDRKSAKE L-322
NtMKK1	190-HRDLKPSNLLI N-201	206-VKITDFGVS-214	220-T SGL ANT-226	233-YMSPER-238	249-DIVSLG-254	263-GVFPY-267	312-QKDQKDRLSANE L-324
OsMKK1	191-HRDI KPSNLLVN-200	207-VKITDFGVS-215	221-SMGQRDT-227	234-YMAPER-239	250-DIVSLG-255	264-GRFPY-268	312-QKDPAERMSASE L-324
BvMKK2	189-HRDLKPSNLLI N-200	205-VKITDFGVS-213	219-TS AGANT-225	232-YMAPER-237	248-DIVSFG-253	262-GKFPY-266	311-QKDPKARHSAQQL-323
BdMKK2	185-HRDLKPSNI LI N-196	201-VKISDFGVS-209	214-SS AGRDT-221	228-YMAPER-233	244-DIVSLG-249	258-GNFPY-262	301-QKEATDRSSAQV L-313
LeMKK2	185-HRDI KPSNLLI N-196	201-VKIADFGVS-209	214-TM DPCNS-221	228-YMSPER-233	249-DIVSLG-254	263-GRFPY-267	307-QRDPARRVTAGQL-319

(B)

(C)

Fig. 1 Alignment and phylogenetic analysis of CaMKK1 and other MAPKK family members and expression patterns of *CaMKK1* in *C. album* under salt and drought stress. **a** Alignment of CaMKK1 amino acid sequence with the representative closely related amino acid sequences-AtMKK1 (AEE85154.1), NtMKK1 (AAF67262.1), OsMKK1 (AAG40578.1), LeMKK (NP_001234588.1), BdMKK2 (NP_001289805.1) and BvMKK2 (XP_010696552.1) using DNA-MAN software. The conserved subdomains are shown with Roman numbers (I–XI) at the top of the sequences, the numbers on both ends of the conserved sequence represent the starting and ending sites of amino acid in the complete sequence. **b** The phylogenetic tree between CaMKK1 and other plant MAPKK proteins. CaMKK1 is in bold and with a dot in front. Different groups (**A, B, C, D**) of MAPKKs are marked on very beginning of the branch on the left. Numbers above or below branches indicate bootstrap values from 1000 replicates. The protein sequences of the MAPKKs used for construction of the tree are acquired from GenBank database. The acces-sion numbers are listed below: AtMKK1 (AEE85154.1), AtMKK2 (AEE85679.1), AtMKK3 (AED94548.1), AtMKK4 (AEE32696.1), AtMKK5 (AEE76478.1), AtMKK6 (AED96783.1), AtMKK7 (AEE29706.1), AtMKK8 (NP_187274.1), AtMKK9 (AEE35470.1), NtMKK1(AAF67262.1), NtMKK2 (AAG53979.1), OsMKK1 (AAG40578.1), OsMKK6 (ABG45894.1), ZmMKK3 (AET74070.1), GmMKK2 (NP_001236577.1), GhMKK2 (ADI52621.1), BnMKK1 (ADZ75456.1), BvMKK2 (XP_010696552.1), BdMKK2 (NP_001289805.1), BdMKK6.1 (AFS18269.1) and LeMKK (NP_001234588.1). **c** Expression patterns of *CaMKK1* and *CaMPK* in *C. album* and *NtMPK4* in transgenic tobacco line (OE3) under salt and drought stress by quantitative RT-PCR. Total RNA was isolated from *C. album* fresh leaves at the indicated treatment time points. NaCl: NaCl treatment; PEG: polyethylene glycol 6000 treatment. Values are means ± SE of eight replicates. Different lowercase letters above the columns indicate significant difference ($P < 0.05$) according to Duncan's multiple range test

component-MPK expression in *C. album* and transgenic tobacco.

Overexpression of *CaMKK1* enhances salt tolerance to bacterium and transgenic tobacco

To investigate the function of salt tolerance, *CaMKK1* was over-expressed in tobacco. Genomic PCR and RT-PCR results showed that *CaMKK1* was transformed into tobacco plants and expressed at transcriptional level (Fig. 2a, left and middle), moreover, the expression level of *CaMKK1* in different transgenic lines (OE1, OE2, OE3) was slight different, i.e. OE3 was the higher, OE1 was in the middle, OE2 was the lower (Fig. 2a, right). Three transgenic lines which exhibited 3:1 segregation ratio between the antibiotic-resistant and non-resistant seedlings were selected for further experiments.

Before the transgenic tobacco lines were analyzed, the performance of ectopic expression of *CaMKK1* in *E. coli* was tested. The time course of growth of recombinant *E. coli* strain harboring with pET-28a-*CaMKK1* was measured under NaCl treatment (Fig. 2b). Results showed that the cells grew similarly well under non-stress condition, whereas the recombinant strain grew much better than that of the control strain when being applied with salt stress (50–200 mM NaCl).

To further understand the role of CaMKK1 in response to stress, we generated transgenic tobacco lines of *CaMKK1*. Seed germination and seedling growth of T2 generation revealed that no significant difference was observed between NT and transgenic lines without NaCl treatment (Fig. 2c), however, with NaCl concentration rising, the germination percentage of three T2 transgenic tobacco lines (OE1, OE2, OE3) was significantly higher than that of NT plants ($P < 0.05$) (Fig. 2d). The seedling and root growth of transgenic lines were significantly greater than that of NT plants in the presence of 100–300 mM NaCl (Fig. 2e, f).

Overexpression of *CaMKK1* confers drought tolerance to bacterium and transgenic tobacco

To gain a better understanding of *CaMKK1* in drought tolerance, we carried out a series of experiments to investigate the performance. First, recombinant *E. coli* strain harboring with pET-28a-*CaMKK1* was measured under PEG treatment (Fig. 3a). Compared to the control, the recombinant strain could tolerate much broader range of different stresses and grow much better when being applied with drought stress.

Furthermore, seed germination was conducted under PEG 6000 (5, 10, 15%) to mimic drought stress. No obvious difference existed between NT and transgenic lines without

PEG treatment (Fig. 3b). However, when treated with PEG, the germination percentage of OE lines were significantly higher than that of NT plant ($P < 0.05$) (Fig. 3c). The root length in OE lines was greater compared to that of NT plants in the presence of PEG (Fig. 3d, e).

To further investigate the drought tolerance of transgenic plants, water loss assay with fresh leaves of 3-month-old plants was performed. Lower water loss rate was detected in transgenic leaves in comparison with the control under dehydration treatment (Fig. 3f), and NT leaves appeared to be more withered than that of transgenic tobacco leaves after dehydration in air for 4 h (Fig. 3g). These results indicate that overexpression of *CaMKK1* can improve the drought tolerance in transgenic tobacco.

Organic osmoprotectants contribute to salt and drought tolerance of *CaMKK1* transgenic tobacco lines

The elevated tolerance of *CaMKK1* transgenic plants was correlated to changes of the physiological and biochemical indexes. In the present study, we found that low level of free proline was detected and no significant difference observed between NT and OE lines under normal condition. However, salt and drought stresses promoted the accumulation of free proline, especially in transgenic plants under 15% PEG stress (Fig. 4a). Soluble sugar content of some transgenic line was significantly higher than that of the NT plant under 15% PEG or 300 mM NaCl treatment (Fig. 4b). The glycine betaine content of transgenic lines was significantly higher than that of NT plants under 15% PEG rather than NaCl treatment (Fig. 4c). There had no significant change of the total soluble protein in NT or transgenic plant under PEG or NaCl treatment (Fig. 4d). These data indicate that overexpression of *CaMKK1* may increase the accumulation of organic osmoprotectants, which should contribute to enhance the drought or salt tolerance.

Overexpression of *CaMKK1* in tobacco alleviated ROS accumulation under drought or salt stress

To investigate whether *CaMKK1* expression can elevate tolerance to oxidative stress or not, in the present study, DAB and NBT staining were used to measure the accumulation of H_2O_2 and O_2^- in freshly detached leaves from NT and OE lines under PEG and NaCl treatment. Under normal condition, DAB staining (H_2O_2 content) had no significant difference between NT and OE lines. Following treatment with NaCl or PEG, the DAB staining was found to be much lighter in the transgenic plants than that of the control plants, which means that the level of H_2O_2 was considerably lower in the transgenic plants (Fig. 5a). The NBT staining (for O_2^- content) of three OE lines was significantly weaker than that of NT plants upon NaCl or PEG stress,
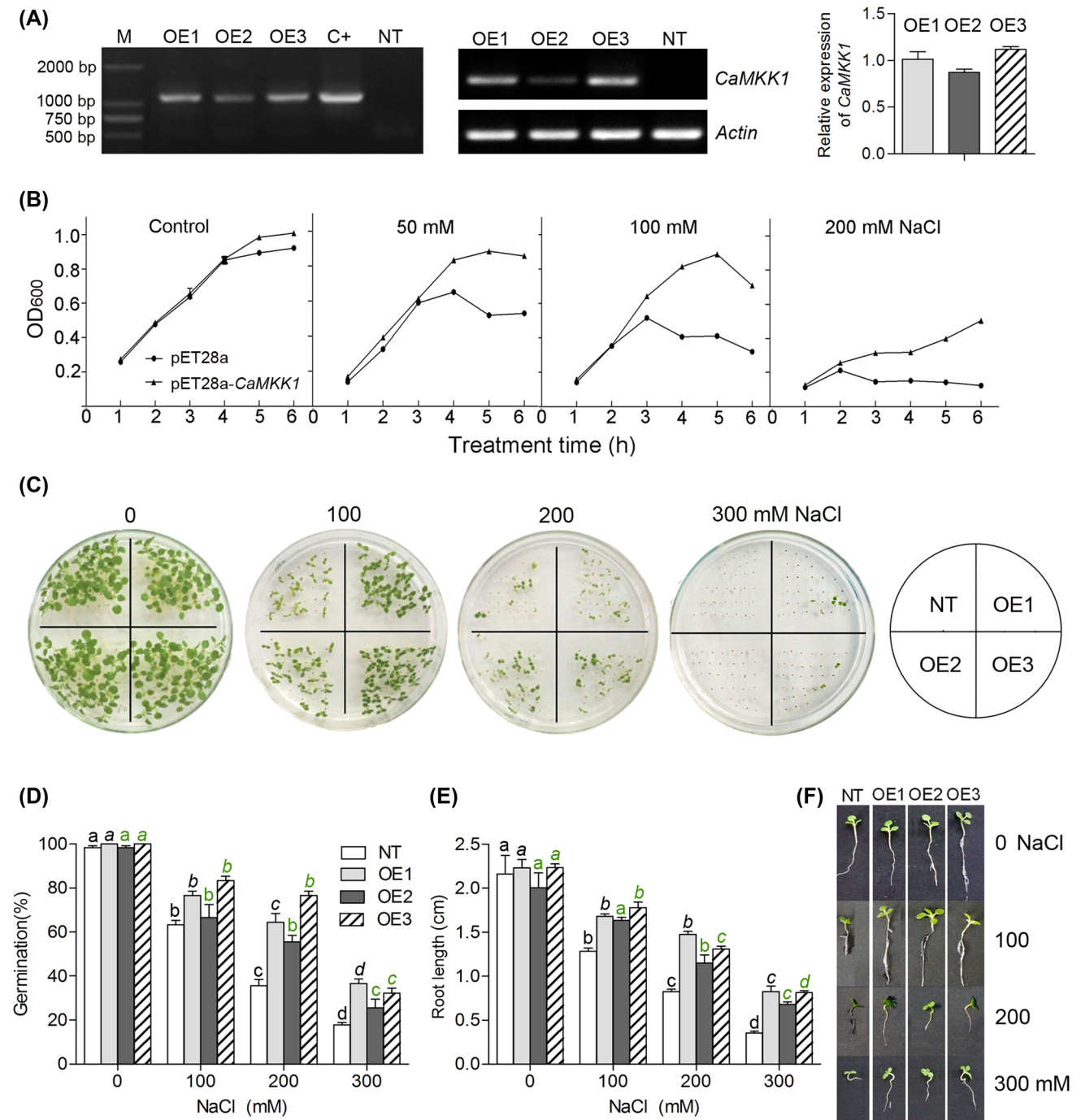
Fig. 2 Overexpression of *CaMKK1* enhances salt tolerance in transgenic tobacco. **a** PCR, RT-PCR, or qPCR analysis of *CaMKK1* transgenic tobacco. *Left* Genomic DNA PCR, *middle* RT-PCR, *Right* qPCR. *M* DL2000, *1–4* T1 transgenic tobacco lines, *C+* positive control, *NT* non-transgenic tobacco plant. **b** Effect of NaCl stress on the growth of recombinant *E. coli* strain pET28a-*CaMKK1* (BL21). **c** Seed germination of NT and T2 transgenic tobacco lines (OE1, OE2, OE3) on MS medium containing different concentrations of NaCl. OE1, OE2, OE3: T2 generation transgenic tobacco lines 1, 2,

3. **d** Germination percentage of NT and T2 transgenic lines on MS medium containing different concentrations of NaCl. **e** Root length of 12 days-old seedlings on MS medium containing different concentrations of NaCl. **f** Seedling development of NT and T2 transgenic lines on MS medium containing different concentrations of NaCl. In (**d**) and (**f**), values are means ± SE of four (germination) or ten (root) replicates. *Different lowercase letters* above the columns indicate significant difference ($P < 0.05$) according to Duncan's multiple range test

which revealed that there was less O_2^- accumulation in OE lines after treatment with NaCl or PEG (Fig. 5b). These data indicate that the transgenic plant accumulates lower level of ROS, and overexpression of *CaMKK1* can either inhibit ROS production or stimulate ROS scavenging.

To quantitatively analyze the accumulation of ROS in plant, we measured H_2O_2 and O_2^- level in transgenic lines under drought and salt stress. Results showed that H_2O_2 level or O_2^- production in NT increased significantly than that of OE lines under NaCl or PEG treatment (Fig. 5c). MDA content of OE lines and NT plants all increased under stress, there were a 87% increase of MDA content under 300 mM NaCl and a 71% increase under 15% PEG stress of NT plants, which were significantly higher than that of OE lines (50% and 33%) (Fig. 5c). Moreover, the activity of antioxidant enzymes-SOD, POD and CAT increased significantly in some of the transgenic lines in comparison to that of NT plants under NaCl or PEG treatment (Fig. 5d). These data suggest that overexpression of *CaMKK1* in transgenic tobacco may result in ROS effective scavenging by regulating the activity of antioxidant enzymes.

Overexpression of *CaMKK1* enhances net photosynthetic rate to transgenic tobacco

The net photosynthetic rate (Anet) and intercellular CO_2 concentration (Ci) of transgenic lines and NT plants were measured to clarify the improvement of the photosynthesis. Before NaCl or PEG treatment, the Anet and Ci showed no significant difference between NT plants and transgenic lines. After treatment, the Anet was significantly increased in OE1 and OE3 compared to NT plants; while the Ci was significantly lower in transgenic lines (except for OE2) than that of NT plants (Fig. 6).

Overexpression of *CaMKK1* enhances stress-responsive genes expression

To investigate the effects of *CaMKK1* overexpression on the downstream genes, we performed quantitative RT-PCR analysis with some typical stress responsible genes: *NtDREB2, NtDREB3, NtDREB4*, a class of DREB/CBF transcription factors that bind to drought response *cis*-element; *LEA*5, *SOD, ADC*2 (stress-related genes). When exposed to NaCl stress, overexpression of *CaMKK1* in transgenic tobacco lines (especially in OE3) dramatically enhanced the expression level of *NtDREB2* with 100-fold more at 24 h ($P < 0.0001$) and 150-fold more at 48 h ($P < 0.0001$), *NtDREB3* almost 20-fold at 24 h ($P < 0.0001$) and 125-fold at 48 h ($P < 0.0001$), and *NtDREB4* 10-fold at 24 h ($P < 0.0001$) and 83-fold at 48 h ($P < 0.0001$) compared to NT plants; under PEG treatment, the increased expression levels of *CaMKK1* in OE lines with

100-fold at 24 h ($P < 0.0001$) in *NtDREB2* and 50-fold at 48 h ($P < 0.0001$), 50-fold at 24 h or 48 h ($P < 0.0001$) of *NtDREB3*, 47-fold at 24 h ($P < 0.0001$) and ninefold at 48 h ($P < 0.0001$) of *NtDREB4* were observed, which suggests that these DREB transcription factors may be regulated by MAPKK and contribute to enhancement of stress tolerance (Fig. 7).

The expression of *NtSOD, NtADC2* and *NtLEA5* in transgenic lines had no significant change compared to that of the control under normal condition. However, the expression of *NtSOD* significantly elevated in the transgenic lines compared to NT plants under NaCl or PEG treatment for 48 h (Fig. 7) ($P < 0.0001$ for NaCl; $P = 0.0009$ for PEG). The expressions of *NtADC2* ($P = 0.0018$ for NaCl and $P = 0.0028$ for PEG) and *NtLEA5* ($P = 0.0269$ for NaCl and $P = 0.0349$ for PEG) in transgenic lines were also significantly higher than that of NT plant under NaCl or PEG treatment for 48 h.

Discussion

A large amount of evidence indicates that MAPK modules are closely related to plant adaptability to environmental stresses (Sun et al. 2014). As a signal convergence point in the hierarchical organization of MAPK cascades, MAPKK plays crucial role in integrating and passing signals, finally transferring extracellular stimuli into intracellular responses (Xu et al. 2016). In the present study, we characterized the stress tolerance function of a group A *MAPKK* from *C. album* (shortened as *CaMKK1*). Based on the molecular cloning and identifying *CaMKK1*, overexpression of which could efficiently scavenge the excess ROS, and improve various stress-responsive physiological parameters in transgenic tobacco under stress conditions, e.g. increased content of osmoprotectants, activity of antioxidant enzymes, net photosynthesis rate, etc. It is noteworthy that transcription factor genes-*DREB2, DREB3, DREB4* in *CaMKK1* transgenic tobacco were remarkably up-regulated in response to salt and drought stress. These findings not only provide the indication of involvement of CaMKK1 in abiotic stress response, but suggest a crosstalk in the signaling pathway between MAPK cascade and ROS signaling.

Phylogenetic analysis of amino acid sequences in our work revealed that CaMKK1 belongs to group A MAPKK and possesses all 11 conserved domains or motifs. It has been reported that AtMKK1, AtMKK2, and AtMKK6 (group A MAPKK) participate in defense response and mediate phytohormone (e.g. ABA, GA) signaling transduction, to increase the tolerance of abiotic stress (Gao et al. 2008; Xing et al. 2009). OsMKK1 (group A) or OsMKK6 (group A) is involved in the regulation of drought or
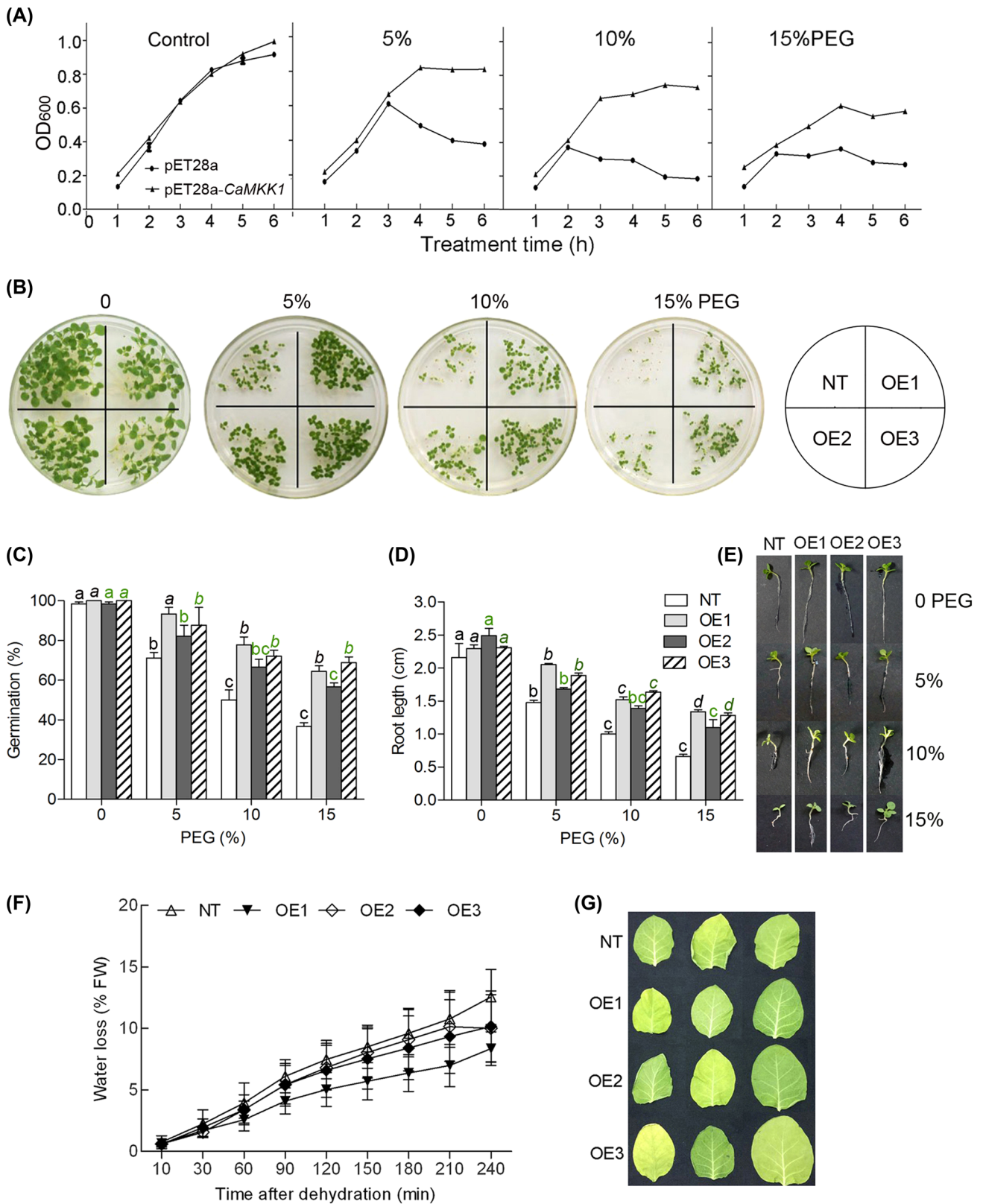
salinity stress tolerance (Kumar et al. 2008). Besides positively response to salt and drought stress, cotton GhMKK1 (group A) also mediates defense response to pathogen infection in transgenic tobacco (Lu et al. 2013). These previous reports suggest a role of group A MAPKKs in plant stress responses.

◄**Fig. 3** Overexpression of *CaMKK1* confers drought tolerance to transgenic tobacco. **a** Effect of PEG stress on the growth of recombinant *E. coli* strain pET28a-*CaMKK1* (BL21). **b** Seed germination of NT and T2 transgenic lines on MS medium containing different concentrations of PEG. *NT* non-transgenic tobacco, *OE1, OE2, OE3* T2 transgenic tobacco lines 1, 2, 3. **c** Germination percentage of NT and T2 transgenic lines on MS medium containing different concentrations of PEG. **d** Root length of 12 days-old seedlings of NT and T2 transgenic lines on MS medium containing different concentrations of PEG. **e** Seedling development of NT and T2 transgenic lines on MS medium containing different concentrations of PEG. **f** The water loss rate. The rate of water loss was calculated by the loss of fresh weight of the samples. **g** The phenotype of detached leaves of NT and transgenic lines at the indicated times. In (**c**) and (**d**), values are means ± SE of four (germination) or ten (root) replicates. *Different lowercase letters* above the columns indicate significant difference ($P < 0.05$) according to Duncan's multiple range test

Salinity and drought are major abiotic factors which affect plant growth and development (Liu et al. 2015). MAPK signaling pathway is well-known in mediating stress-responsive signaling (Colcombet and Hirt 2008). In the present study, *CaMKK1* (the crucial component in the MAPK cascade) was up-regulated under salinity and drought stress in *C. album*. It is consistent with the reports of *TaMAPKK* in *T. aestivum* and *LcMKK* in *L. chinense* (Wen et al. 2015; Wu et al. 2015); whereas *AtMKK9* (*A. thaliana*), *BdMKK6.2* (*B. distachyon*) or *GhMKK5* (*G. hirsutum*) applied negative effect at the same conditions (Xu et al. 2008; Zhang et al. 2012; Sun et al. 2016). It is suggested that different members of *MAPKKs* may play diverse roles in response to stress (Colcombet and Hirt 2008). In the present study, ectopic expression of *CaMKK1* in prokaryote displayed much better growth under salt and drought stress; moreover, *CaMKK1* overexpression in tobacco not only improved seed germination and seedling growth, but also enhanced the expression of downstream gene-*NtMPK4* in transgenic lines under stress condition. Similar result shows that overexpression of *ZmMKK4* in Arabidopsis had a significantly higher germination rate and better seedling growth under salt stress (Kong et al. 2011), while a T-DNA insertion mutation of Arabidopsis *MKK2* gene confers hypersensitivity to salt stress in germination (Teige et al. 2004). In addition, MAPK is the last component of MAPK cascade and plays crucial roles in linking upstream MAPKKs and downstream components in stress signaling (Agarwal et al. 2010; Wang et al. 2015). In the present study, we found that *CaMPK* in *C. album* and *NtMPK4* in *CaMKK*-overexpressed transgenic tobacco could actively respond to salt and drought stress, and all of them displayed similar fast and transient transcription pattern. Taken together, it suggests that CaMKK1 should be a positive and important regulator in MAPK cascade in response to salt and drought stress.

Proline and soluble sugars play an important role in adaptation to adversity in plants. When countering the environmental stimulations, proline in particular increases the water-control capacity and helps to prevent water loss, avoid protein denaturation, and minimize disruption to cellular metabolism (Liu and Zhu 1997). Accumulation of soluble sugars and glycine betaine not only reduces the solute potential in plant cells, but also assists the scavenging and regulation of ROS (Couée et al. 2006; Fan et al. 2012). In the present study, water loss rate was lower in the transgenic tobacco lines than NT plants under dehydration treatment, and the compatible organic molecules-proline, soluble sugar and glycine betaine in *CaMKK1*-overexpressed tobacco lines were significantly accumulated compared to that of NT plants. This result was corresponding to the improved net photosynthesis rate of transgenic tobacco lines in our experiment. Avoiding excessive water loss is very important for various physiological activities, especially the photosynthesis (Chaves et al. 2009). Our results suggest that overexpression of *CaMKK1* in tobacco protects transgenic plant from losing water probably by accumulating the osmoprotectant content, which then contributes to improving photosynthesis and adaptation to salt and drought stress.

It has been reported that ROS can activate MAPK, whereas disturbing MAPK cascades can modulate ROS production and action (Liu and He 2017). In the present study, we found that overexpression of *CaMKK1* significantly decreased O_2^-, H_2O_2 and MDA accumulation in transgenic lines under salt and drought stress, which was corresponding to the histochemical staining of DAB or NBT with a significant reduction of H_2O_2 or O_2^- in transgenic lines. ROS and MAPK-associated signaling pathways play a crucial role in stimulating the response to protect against oxidative stress (Liu and He 2016). A great deal of research has shown that induction of the antioxidant enzymes is important for protection against various stresses (Sinha et al. 2011). Overexpression of *AtMKK1* or *AtMKK2* adjusts ROS metabolism by regulating the catalase genes which encode the H_2O_2 scavenging enzymes (Pitzschke et al. 2009). Our further analysis showed that overexpression of *CaMKK1* alleviated the accumulation of ROS by improving the activity of ROS scavenging enzymes-SOD, POD and CAT under salt and drought stress. Our results were consistent with earlier reports on *MAPKK* genes in other plants (Kong et al. 2011; Lu et al. 2013; Wu et al. 2015). SOD is the first line of defense against ROS (Samuel and Ellis 2002) by catalyzing the dismutation of O_2^- to oxygen and H_2O_2, which plays an important role in protecting cells against superoxide radicals. In our study, in accord to the enzyme activity of SOD, the transcription level of *NtSOD* in transgenic tobacco increased significantly compared to that of NT plants under stress condition. Our data suggest that CaMKK1 can regulate the expression and activity of these antioxidant enzymes, which may lead to
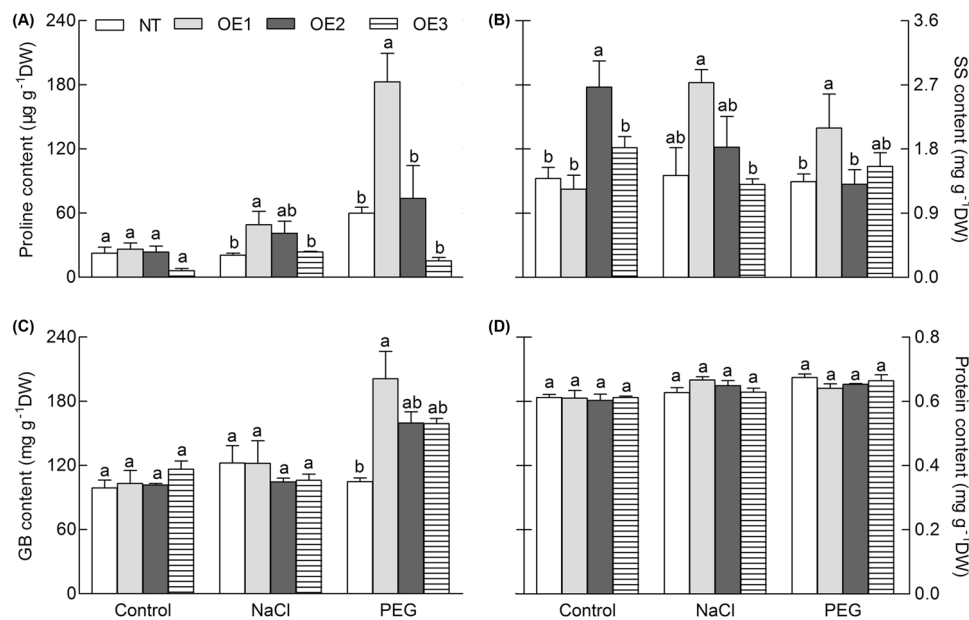
Fig. 4 Analysis of organic osmoprotectants in transgenic tobacco. **a** Proline content, **b** soluble sugar content, **c** betaine content, **d** protein content of NT and transgenic lines under NaCl and PEG treatment. *NT* non-transgenic tobacco, *OE1, OE2, OE3* T1 transgenic tobacco lines 1, 2, 3. Values are means ± SE of ten replicates. *Different lowercase letters* above the columns indicate significant difference (*P* < 0.05) according to Duncan's multiple range test

enhancement of ROS scavenging and protection against salt and drought stresses.

The dehydration response element binding proteins are important transcription factors in regulation of plant response to abiotic stresses (Kamioka et al. 2016). In the present study, three transcription factors-*NtDREB2, NtDREB3, NtDREB4* were sharply up-regulated in transgenic tobacco lines compared to that of NT plants under NaCl or PEG treatments, i.e. at least 15-fold and at most near 224-fold (with an average of more than 30-fold) of the transcript accumulation of *NtDREB2, NtDREB3, NtDREB4* were induced. Compared to other genes in the present study, the *DREB* genes responded to the stress much quicker and stronger in *CaMKK1*-overexpressed tobacco. It suggests that MAPKK might regulate the expression of DREB transcription factors directly in the MAPK signaling pathway in response to stress. It has been reported that *NtDREB2A* was up-regulated in *ZmMKK4*-overexpression transgenic tobacco under salt stress (Kong et al. 2011). *LEA5* is a group 5 late embryogenesis abundant protein, which can accumulate substantially in plants under high-salt stress and dehydration (Liu et al. 2009). In the present study, *NtLEA5* was up-regulated in transgenic tobacco lines compared to that of NT plants under stress. Arginine decarboxylase (*ADC*) is a rate-limiting enzyme that catalyzes the first step of polyamine biosynthesis, which is involved in abiotic stress responses, e.g. salinity, heat and low temperature (Urano et al. 2005). The expression of *NtADC2* in the

present study was significantly induced in transgenic lines under stress. The high expression of these stress-related genes should contribute to salt and drought tolerance in *CaMKK1*-overexpressed tobacco lines, and which might be involved in the regulation of the signaling from CaMKK1 to DREBs.

The significant involvement of MAPKKs in ROS-related signaling pathways had been described previously. Stimuli cause the activation of AtMEKK1, then activate AtMEK1-AtMPK4 module, which is the first identified cascade pathway in drought response (Ichimura et al. 1998; Mizoguchi et al. 1998). Later, MEKK1-MKK2-MPK4/MPK6 module related to enhancement of salt tolerance is characterized (Matsuoka et al. 2002; Teige et al. 2004). In the present study, CaMKK1 shared high similarity with AtMKK1 and AtMKK2 in group A, based on which we speculate that the MAPK cascade in *C. album* responds to stress similar to AtMKK1 and AtMKK2 in Arabidopsis. When ROS is generated upon salt or drought stress, which may activate MAPK signal transduction pathway, and the CaMKK1, as a key point of MAPK cascade, may in turn activate the CaMPK, and then modulate the expression of downstream transcription factor (TF) and stress-related genes, e.g. DREB TF. The quick and high expression of *NtDREBs* in *CaMKK1* transgenic tobacco under stress in the present study may present an evidence for our speculation. However, further studies are necessary to characterize MAPK-related cascade of *C. album* in response to abiotic stress.
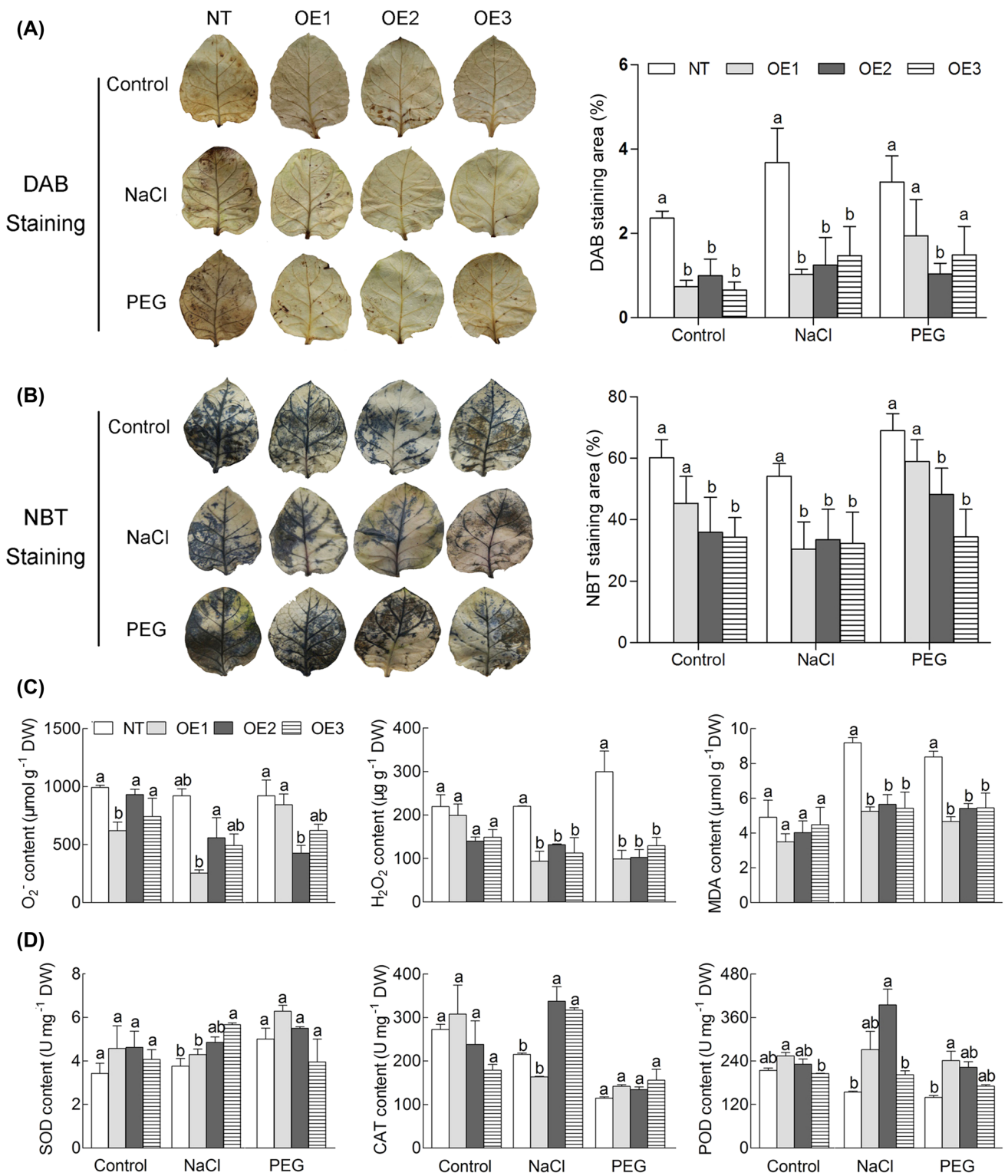
Fig. 5 Analysis of ROS accumulation and activity of antioxidant enzymes in NT and transgenic lines in response to salt and drought stress. **a, b** *Left* histochemical staining by DAB or NBT to reveal accumulation of H_2O_2 or O_2^- in leaves of NT and transgenic lines, *right* statistical analysis with the Image G and GraphPad Prism 5.0. Staining area (%) represents the ratio between the stained area and the whole leaf area. **c** Changes of ROS level and MDA content and **d** the activity of antioxidant enzyme in NT and transgenic lines after treatment with NaCl and PEG. *NT* non-transgenic tobacco, *OE1, OE2, OE3* T1 transgenic tobacco lines 1, 2, 3. Values are means ± SE of four replicates. *Different lowercase letters* above the columns indicate significant difference ($P < 0.05$) according to Duncan's multiple range test

Fig. 6 Analysis of net photosynthetic rate (Anet) and the intercellular CO_2 concentration (Ci) in NT and transgenic lines in response to salt and drought stress. **a** NaCl treatment. **b** PEG treatment. *NT* non-transgenic tobacco, *OE1, OE2, OE3* T1 transgenic tobacco lines 1, 2, 3. Values are means ± SE of ten replicates. *Different lowercase letters* above the columns indicate significant difference ($P < 0.05$) according to Duncan's multiple range test
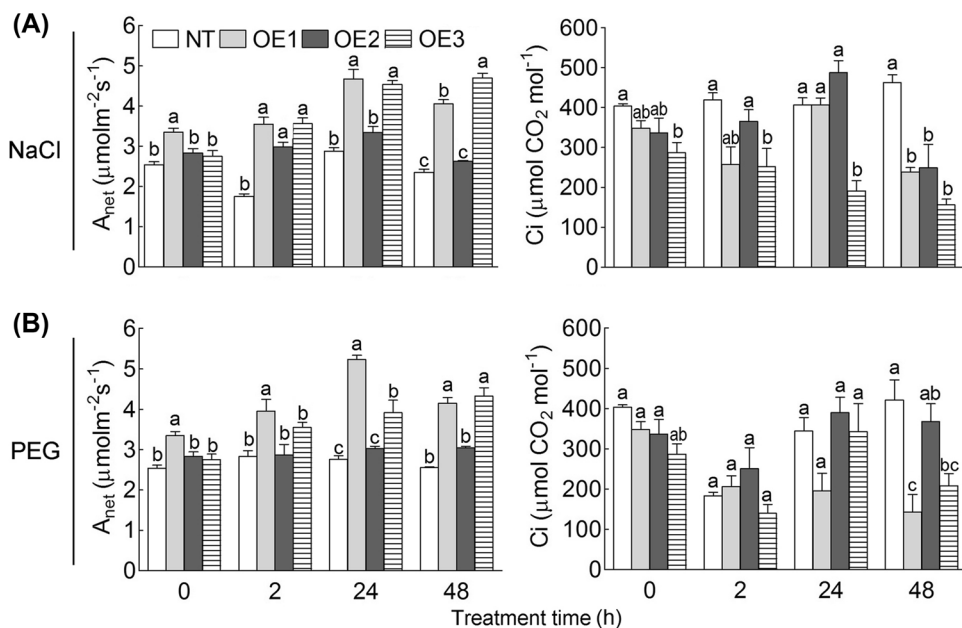
Fig. 7 Expression patterns of stress-related genes under NaCl and PEG treatment in NT and transgenic lines by quantitative RT-PCR. **a** NaCl treatment. **b** PEG treatment. *NtDREB2, NtDREB3, NtDREB4* dehydration response element binding factor 2/3/4, *NtSOD* superoxide dismutase, *NtADC2* arginine decarboxylase, *NtLEA5* group 5 late embryogenesis abundant protein. These genes are all from *N. tabacum*. *NT* non-transgenic tobacco, *OE1, OE2, OE3* T1 transgenic tobacco lines 1, 2, 3. Values are means ± SE of eight replicates. *Different lowercase letters* above the columns indicate significant difference ($P < 0.05$) according to Duncan's multiple range test
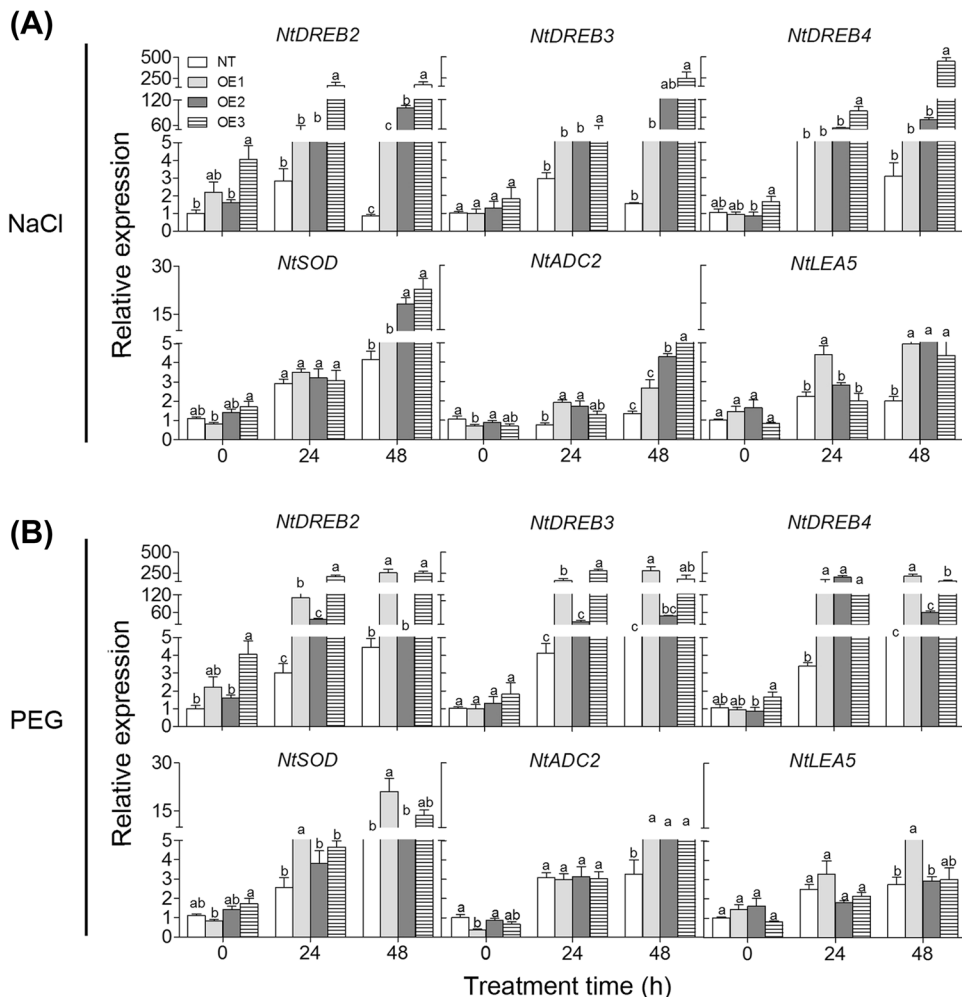
Conclusion

A group A MAPKK gene-*CaMKK1* was identified and characterized from a salt-tolerant species-*C. album*. Overexpression of *CaMKK1* in prokaryote or in transgenic tobacco lines improved the salt and drought tolerance, which reflects in the transgenic tobacco lines with significant improvement of physiological activities, e.g. increased proline, soluble sugar or glycine betaine contents; reduced water loss and improved net photosynthesis rate, etc. under salt and drought stresses. Besides, the improved stress tolerance of *CaMKK1* overexpressed lines could partially be related to activation of antioxidant genes/enzymes, which lead to a more efficient scavenging of ROS under stress conditions. Meanwhile, stress-responsive genes were upregulated by *CaMKK1* overexpression, especially the DREB transcription factors. *NtDREB2, NtDREB3, NtDREB4* quickly and dramatically responded to stress in transgenic tobacco, suggesting that CaMKK1 may transfer signal to CaMPK which then interacts with downstream TF components. The results of the present study suggest that *CaMKK1* acts as a positive regulator in stress responsible signaling pathway. The present study will no doubt broaden our knowledge on further understanding of the molecular mechanism of MAPK signal transduction pathway in response to stress.

Author contributions HYL designed the project and the experiments. JW, XXL, SXJ, YLM, SYZ, YL and XRL performed the experiments. JW, XXL and SXJ analyzed the data. JW and HYL wrote and revised the manuscript. All authors read and approved the manuscript.

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