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Overexpression of maize chloride channel gene *ZmCLC-d* in *Arabidopsis thaliana* improved its stress resistance

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Abstract

In plant cells, anion channels and transporters are essential for key functions. Members of the chloride channel (CLC) family located in intracellular organelles are required for anion accumulation, pH adjustment, and salt tolerance. Here, we cloned a maize (*Zea mays* L.) CLC gene, named *ZmCLC-d*, and found that its transcription was up-regulated under cold, drought, salt, and heat stresses, and after hydrogen peroxide (H_2O_2) and abscisic acid (ABA) treatments. The overexpression of *ZmCLC-d* in *Arabidopsis* conferred tolerance to cold, drought, and salt stresses; this tolerance was primarily displayed by an increased germination rate, root length, plant survival rate, antioxidant enzyme (catalase, peroxidase, and superoxide dismutase) activities, and a reduced accumulation of Cl^- in transgenic plants as compared with wild type (WT) plants. The accumulation of H_2O_2 and superoxide anion in leaves of the *ZmCLC-d*-overexpressing plants is much less than that of the WT plants. The expressions of some stress related genes, such as *CBF1*, *CBF2*, *CBF3*, *DREB2A*, and *RCI2A*, increased to a greater extent in the *ZmCLC-d*-overexpressing plants than in the WT. Our results strongly suggest that *ZmCLC-d* played an important role in stress tolerance.

Additional key words: catalase, cold, heat, H_2O_2, peroxidase, superoxide dismutase, water stress, *Zea mays*.

Introduction

Chilling stress is a major environmental factor that limits agricultural productivity. Chloride channel proteins comprise a protein family that mediates chloride transport across the plasma membrane and intracellular membranes. The chloride channel (CLC) family are ubiquitous proteins present in prokaryotes and eukaryotes, and, unusually, comprise both channel proteins and transporters (Mindell and Maduke 2001, Jentsch 2008). The roles of plant anion channels include nutrient homeostasis, signaling, and responses to biotic and abiotic stresses (Isayenkov *et al.* 2010). Seven *AtCLC* genes (*AtCLCa-g*) have been identified in the *Arabidopsis* genome (Hechenberger *et al.* 1996, Lv *et al.* 2009). Intracellular localization of some of these proteins shows that AtCLCs are present in various membranes including the vacuolar membrane (AtCLCa and AtCLCb) (De Angeli *et al.* 2006, Von der Fecht-Bartenbach *et al.* 2010), Golgi vesicles (AtCLCd and AtCLCf), or chloroplast membranes (AtCLCe) (Marmagne *et al.* 2007, Von der Fecht-Bartenbach *et al.* 2007). Physiological characterization of *Arabidopsis* mutants suggested the involvement of AtCLCa, AtCLCb, and AtCLCc in the regulation of nitrate content in plants (Geelen *et al.* 2000, Harada *et al.* 2004, Von der Fecht-Bartenbach *et al.* 2010). AtCLCa is located at the tonoplast in *Arabidopsis* mesophyll cells and functions as NO_3^-/H^+ antiporter. In agreement, an *AtCLCa* knockout mutant accumulates 50 % less nitrate than wild-type plants (Geelen *et al.* 2000, De Angeli *et al.* 2006). Thus, AtCLCa plays a major role in nitrate homeostasis, probably partly in cooperation with AtCLCe which is localized in the chloroplast (Monachello *et al.* 2009). It has also been proposed that AtCLC proteins located in cellular organelles participate in the establishment of acidic intra-organellar pH. AtCLCd has been shown to be targeted to the trans-Golgi network (TGN), to co-localize with V-type ATPase, and to be involved in adjustment of the luminal pH of this compartment (Von der Fecht-Bartenbach *et al.* 2007).

AtCLCa plays a role in the translocation of nitrate

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Abbreviations: ABA - abscisic acid; CAT - catalase; CLC - chloride channel; H_2O_2 - hydrogen peroxide; MDA - malondialdehyde; O_2^- - superoxide anion; POD - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase; TGN - trans-Golgi network.

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into the vacuole, but all the other characterized CLCs in bacteria and mammals are involved in chloride transport. It was recently shown that this difference in selectivity is due to an amino acid change in the selectivity filter (Bergsdorf *et al.* 2009, Wege *et al.* 2010). Three of other AtCLCs (AtCLCc, AtCLCd, and AtCLCg) possess a selectivity filter in favour of chloride transport (Zifarelli and Pusch 2010). It has been suggested that these proteins could be involved in processes that require Cl^- transport, such as stomatal movement or salt tolerance (Hänsch and Mendel 2009, Teakle and Tyerman 2010). In response to salt stress, the transcription of the chloride channel gene *OsCLC1* is repressed in the salt-sensitive rice line IR29 which accumulates Cl^-; however, the transcription is transiently induced in the salt tolerant rice line Pokkali which excludes Cl^- (Diédhiou and Golldack 2006).

In contrast to the detailed knowledge regarding the molecular regulation of K^+/Na^+ homeostasis and the metabolism of osmo-protective compounds (Sun *et al.* 2010), the regulation of anion transporters and particularly of Cl^- transport systems in plants remain poorly understood. Maize is one of the most important crops worldwide, but its growth and yield are severely limited by high salinity or low temperature (Kizis 2002). It has been reported that *ZmCLC-d* was induced in maize by chilling stress (Yang *et al.* 2011), and the CLC protein in mitochondria is divergently selected for cold tolerance in maize populations (Tampieri *et al.* 2011). In the present study, we showed the expression patterns of the *ZmCLC-d* gene under cold, drought, salt, and heat stresses, and H_2O_2 and abscisic acid (ABA) treatments. We also studied transgenic *Arabidopsis* plants that constitutively expressed *ZmCLC-d* and their responses to multiple stresses.

Materials and methods

To isolate RNA samples for cloning, the seedlings of a maize (*Zea mays* L.) inbred line W9816 were grown up to the three-leaf stage in an incubator at a temperature of 25 °C, an air humidity of 50 %, a photosynthetic photon flux density of about 450 μmol m^{-2} s^{-1}, and a 14-h photoperiod, and then were treated at 4 °C for 24 h. Total RNA was extracted usinga *TRIzol* reagent (*Invitrogen*, Carlsbad, USA). First-strand cDNA was synthesized from 2 μg of total RNA using a cDNA synthesis kit (*Promega*, Madison, USA) according to the manufacturer's instructions. A partial sequence of maize chloride channel d-like (CLC-d) mRNA was identified *via* searching the database of the National Center for Biotechnology Information (*NCBI*) (http://www.ncbi.nlm.nih.gov/ nuccore/GU344733). Full-length *ZmCLC-d* cDNA was obtained by the 5' rapid amplification of cDNA ends (RACE). RACE cDNA was synthesized using a *SMART RACE* amplication kit (*Clontech*, Dalian, China) according to the manufacturer's protocol. The full-length coding regions of *ZmCLC-d* were amplified from cDNA samples to verify the results of RT-PCR and RACE. (Primers, see Table 1 Suppl.).

For further experiments, the seeds of the maize inbred line W9816 were surface-sterilized with 75 % (v/v) ethanol for 3 min and then rinsed three times with distilled water before they were sown in pots containing a transplanting medium consisting of peat, *Vermiculite*, and *Perlite* (10:1:1, v/v/v) moistened with a Murashige and Skoog (MS) medium. The plants were grown in an incubator at 25 °C, an irradiance of 450 μmol m^{-2} s^{-1}, a 14-h photoperiod, and a relative humidity of 50 % until the seedlings developed three leaves. Then the seedlings were subjected to a cold stress (4 °C), drought stress (a MS solution containing 20 % PEG-6000), high salinity (a MS solution containing 500 mM NaCl), heat stress (42 °C), oxidative stress (a MS solution containing 50 mM H_2O_2), and 100 μM ABA for 0, 2, 4, 8, 12, 24, and 36 h. After all the treatments, the seedlings were harvested, frozen in liquid nitrogen and stored at -80 °C for further analyses. Control (untreated) plants were harvested at the same times as the treated plants.

Arabidopsis thaliana L. (ecotype Columbia, Col-0 and *Atclcd-1*) seeds were surface-sterilized, stratified by incubation in the dark at 4 °C for 2 d, and then grown on MS agar plates or soil in a chamber with a 16-h photoperiod, an irradiance of 200 μmol m^{-2} s^{-1}, a temperature of 22 °C, and a relative humidity of 75 %. Three-week-old WT, clcd-1, and *ZmCLC-d*-over-expressing *Arabidopsis* plants were used for observing phenotype. For cold treatment, two weeks old seedlings grown under normal conditions were transferred to 4 °C for 7 d. For drought treatment, two weeks old seedlings grown under normal condition were not watered for two weeks, and then the plants were re-watered for 7 d. For freezing treatment, four weeks old seedlings grown under normal conditions were transferred to -10 °C for 10 h.

Total RNA was extracted from maize or *Arabidopsis* seedlings using a TRIzol reagent (*Invitrogen*) and was treated with RNase free DNase (*Promega*). For real-time quantitative PCR (RT-qPCR), 1.5 μg of total RNA was used to perform it with *PrimeScript RT Master Mix* (*TaKaRa*, Dalian, China). The cDNA samples were diluted to 30 mg dm^{-3}. Triplicate quantitative assays were performed with 0.002 cm^3 of each cDNA dilution with *SYBR Premix ExTaq* (*TaKaRa*) and the *ABI 7500* sequence detection system according to the manufacturer's instructions (*Applied Biosystems*, Carlsbad, USA). The relative quantification method (delta-delta CT) was used to evaluate variations between the examined replicates.

The digestion product *ZmCLC-d* from pMD18-T-*ZmCLC-d* was directionally cloned into *Xba* I and *Sac* I sites of a pCAMBIA3301 vector, which had CaMV35S in *Hind* III and *Xba* I sites and NOS TER in

Sac I and *EcoR* I sites, to generate pCAMBIA3301-*ZmCLC-d*. *ZmCLC-d* was driven by a CaMV35S promoter. The construct was electroporated into *Agrobacterium tumefaciens* strain EHA105. The Col-0 and clcd-1 were transformed by the floral dip method (Clough *et al.* 1998).

Chloride content was analyzed at 3-week-old plants firstly grown on MS agar plates and then transferred to new MS agar plates supplemented with 50 mM NaCl for 24 h. Chlorides were extracted from fresh root tissue (30 mg) using nine volumes of water and three series of freezing (-20 °C) and thawing, and then analyzed by HPLC.

MDA content and activities of catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) in leaves were assayed using a kit obtained from the Nan Jing Jian Cheng Bioengineering Institute according to their instructions. Briefly, leaf segments (0.5 g) were extracted on ice in 5 cm^3 of a 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM ethylenediamine-tetraacetic acid and 1 % (m/v) polyvinylpyrrolidone. The homogenate was centrifuged at 15 000 *g* and 4 °C for 20 min, and the supernatant was immediately used for activity assays. Total POD activity was measured by monitoring the oxidation of 3,3'-dimethoxybenzidine at 470 nm. One unit of POD activity was defined as an increase in absorbance of 1.0 per min. Total CAT activity was assayed by measuring the rate of decomposition of H_2O_2 at 240 nm. Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium. One unit of SOD activity was defined as the amount of enzyme that was required to cause a 50 % inhibition of the reduction of nitro blue tetrazolium as monitored at 560 nm.

H_2O_2 accumulation was detected by the 3,3'-diaminobenzidine (DAB) staining method (Orozco-Cárdenas and Ryan 1999), and O_2^- was detected using the nitroblue tetrazolium (NBT) staining method (Anderson and Greenwald 1985). The seedlings were infiltrated with 5 g dm^{-3} DAB of pH 3.8 for 20 h, and 0.5 g dm^{-3} NBT for 20 h in the dark to detect H_2O_2 and O_2^-, respectively. The seedlings were then decolorized by boiling in 96 % (v/v) ethanol for 10 min. After cooling, the leaves were extracted at room temperature with fresh ethanol and photographed using a stereomicroscope (*Olympus*, Tokyo, Japan).

All values were the means of three assays carried out for each value. Data were tested at significant levels of *P* value < 0.05 using one-way *ANOVA*.

Results

A known incomplete *CLC* gene of maize retrieved from GenBank (accession No. GU344733) was searched against the *NCBI* database. Based on the partial sequence, a pair of primers was designed and used to amplify the fragment from maize seedlings that were treated at 4 °C for 24 h. Subsequently, 5' RACE was conducted to obtain the missing coding region, and then a pair of primers was designed to confirm the sequence of full-length cDNA by RT-PCR. The full length of this gene was 2 619 bp with an open reading frame of 2 355 bp and was predicted to encode a protein of 784 amino acid residues with a calculated molecular mass of 86.42 kDa. The complete mRNA sequence of the *ZmCLC-d* gene has been deposited in the GenBank database and the GenBank acc. No. is KF153239. Analyses of structural properties of the ZmCLC-d predicted protein using *Pfam* (Bateman *et al.* 2004) and *SMART* programs (Letunic *et al.* 2004) suggest that this protein encodes eight putative transmembrane domains (amino acids 108-130, 224-246, 261-278, 313-335, 350-372, 442-461, 466-488, and 500-522), one voltage CLC domain (amino acids 120-547) and two cystathionine-β-synthase (CBS) domains (amino acids 586-638 and 697-745) (Fig. 1*A*). In chloride ion channels, the CBS domains have been implicated in intracellular targeting and trafficking as well as in protein-protein interactions. On the basis of the amino acid sequence alignment with other *CLC* genes, the sequence showed a great similarity (82.9 %) with the AtCLC-d protein sequence. For this reason, we named the maize protein here investigated as ZmCLC-d. The ZmCLC-d sequence showed even a higher similarity with ClCs from other sources, being 91.5 % with OsCLC4 from *Oryza sativa* and 88.7 % with TaCLC-d from *Triticum aestivum* (Table 1).

We used RT-qPCR to examine the relative transcript abundance in various maize tissues and the expression patterns of *ZmCLC-d* under different stresses (Table 2). Generally, *ZmCLC-d* was expressed in leaves, roots, and stems, but relatively more in leaves than in roots and stems. Under the cold stress, the *ZmCLC-d* transcription was maintained constant in the first 4 h and then increased up to the maximum level after 12 h (Table 3). A similar expression pattern was observed under the salinity, however, the expression was strongly induced also after 24 h (Table 3). *ZmCLC-d* was induced within 2 h of the onset of the water stress, and it was strongly expressed after 24 h (Table 3). At the heat stress, the *ZmCLC-d* expression was strongly induced after 24 h. These results demonstrate that *ZmCLC-d* took part in response to these stresses. Applied ABA and H_2O_2 also significantly increased the *ZmCLC-d* expression; the amounts of the transcripts were very high after 8 and 12 h, respectively, and declined thereafter (Table 3). This indicates that ABA and H_2O_2 also played important roles in regulating the *ZmCLC-d* expression in maize.

The full-length *ZmCLC-d* sequence under the CaMV-35S promoter was transformed into a loss of function of the *AtCLC-d* T-DNA insertion line (SALK_042895), *clcd-1*, and Col-0 wild-type *Arabidopsis* plants. Four transgenic lines were randomly selected in which

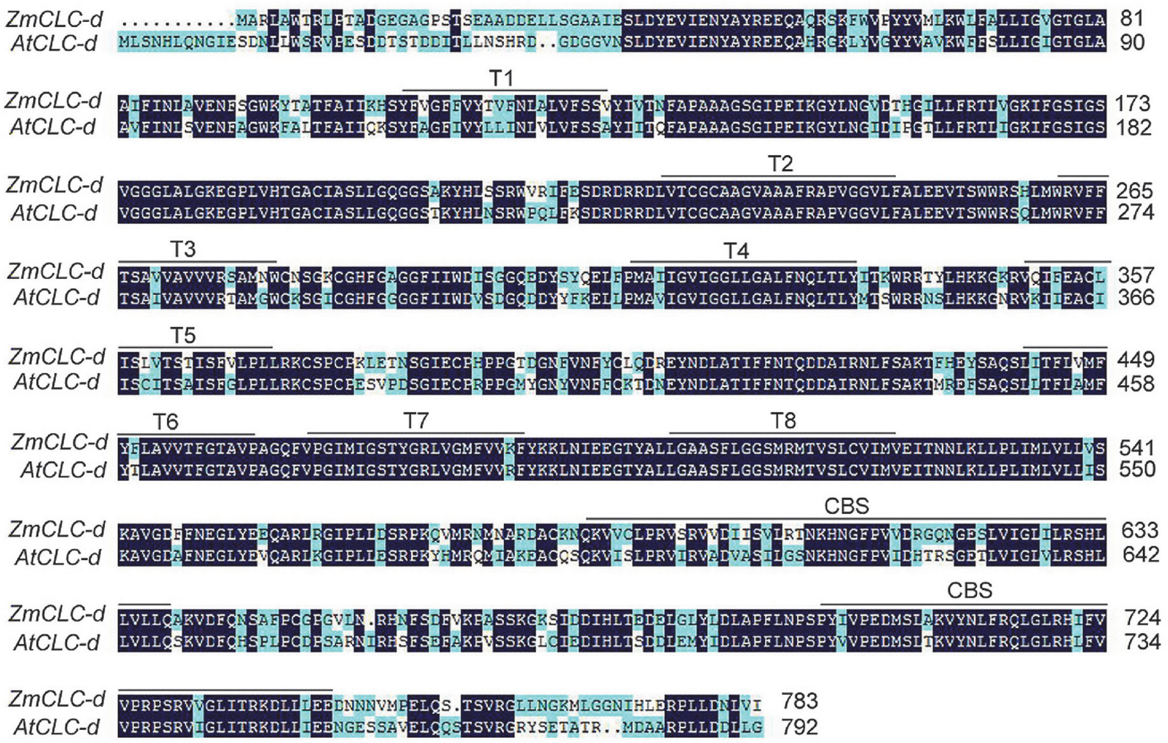

Fig. 1. The multiple alignment of amino acid sequences of *ZmCLC-d* with *AtCLC-d* (At5g26240). T1 - T8 - transmembrane domains, CBS - cystathionine-β-synthase (CBS) domain.

Table 1. Identity and similarity percentages between ZmCLC-d protein and *Arabidopsis thaliana*, *Oryza sativa*, *Glycine max*, *Medicago truncatula*, *Ricinus communis*, *Triticum aestivum*, and *Vitis vinifera* CLC proteins.

CLC protein	Locus	Identity [%]	Similarity [%]
AtCLC-a	At5g40890	38.0	52
AtCLC-b	At3g27170	41.9	57.3
AtCLC-c	At5g49890	43.7	59.0
AtCLC-d	At5g26240	73.7	82.9
AtCLC-e	At4g35440	18.4	32.3
AtCLC-f	At1g55620	17.3	28.9
AtCLC-g	At5g33280	44.9	60.6
OsCLC1	Os01g65500.2	44.0	58.5
OsCLC2	Os01g50860.1	18.7	32.2
OsCLC3	Os02g35190.1	44.9	59.6
OsCLC4	Os03g48940.3	88.5	91.5
OsCLC5	Os04g55210.1	42.8	58.9
OsCLC6	Os08g20570.1	43.5	57.5
OsCLC7	Os12g25200.1	36.6	52.2
OsCLC8	Os08g38980.1	19.2	31.8
OsCLC9	Os02g48880.1	18.6	31.0
OsCLC10	Os04g36560.1	43.8	58.5
GmCLC-d	XP_003517583	74.0	84.0
MtCLC-d	XP_003610692	73.6	84.5
RcCLC-d	XP_002517213	75.6	85.4
TaCLC-d	ADP02177	83.6	88.7
VvCLC-d	XP_002281606	76.2	84.0
ZmCLC-c	AAP04392	45.0	59.6

Table 2. The relative transcript abundance of *ZmCLC-d* in roots, stems, and leaves. *β-Actin* and *GAPDH* were used as internal controls. Means ± SD, *n* = 3.

Organ	*ZmCLC-d*
Roots	1.00 ± 0.06
Stems	1.52 ± 0.08
Leaves	1.76 ± 0.09

ZmCLC-d mRNA was shown by RT-PCR to be highly expressed (Fig. 2*A*). To examine the possible phenotypes of the transgenic lines, the T_3 progeny of the *ZmCLC-d*-overexpressing plants were grown in a greenhouse under identical conditions. Compared with the WT plants, the transgenic and *clcd-1* plants displayed no differences in growth and development under long- or short-day conditions, and germinated and grew normally on agar supplemented with various concentrations of nitrate or chloride (Fig. 2*B*).

To investigate the effect of *ZmCLC-d* overexpression on freezing tolerance, T_3 transgenic, *clcd-1*, and WT seedlings were exposed to -10 °C for 10 h. After recovering for 6 d at 25 °C (Fig. 2*C*), survival was recorded as 11.49 % for the wild type, 10.00 % for *clcd-1*, 72.52 % for 35S::*ZmCLC-d*/*clcd-1*, and 77.22 % for 35S::*ZmCLC-d* (Table 4). Phenotypically, most of the transgenic seedlings were green and could regrow;

Table 3. The expression of *ZmCLC-d* in maize leaves sprayed with 50 mM H_2O_2 or 100 µM abscisic acid, or affected by high salinity (500 mM NaCl), water stress (20 % PEG 6000), heat (42 °C), and cold (4 °C). Means ± SD, *n* = 3, * - significant differences at $P \leq 0.05$ determined by the Tukey's test.

Treatments	0 h	2 h	4 h	8 h	12 h	24 h	36 h
H_2O_2	1.00±0.12	1.06±0.19	4.43±0.64*	3.58±0.45*	14.73±1.98*	9.45±1.05*	6.76±0.87*
ABA	1.00±0.12	1.35±0.11*	1.44±0.09*	1.76±0.17*	0.91±0.16*	0.62±0.05*	0.51±0.06*
NaCl	1.00±0.07	1.11±0.12	2.14±0.21*	3.02±0.29*	4.17±0.36*	10.79±1.03*	6.93±0.58*
PEG	1.00±0.06	3.10±0.33*	2.24±0.15*	5.50±1.82*	8.26±1.13*	22.91±2.19*	13.41±1.83*
Heat	1.00±0.08	1.96±0.13*	3.89±0.12*	4.06±0.22*	9.43±0.24*	16.12±1.55*	7.02±0.10*
Cold	1.00±0.09	1.39±0.06*	1.14±0.03	1.49±0.08*	2.00±0.05*	1.38±0.10*	0.53±0.09

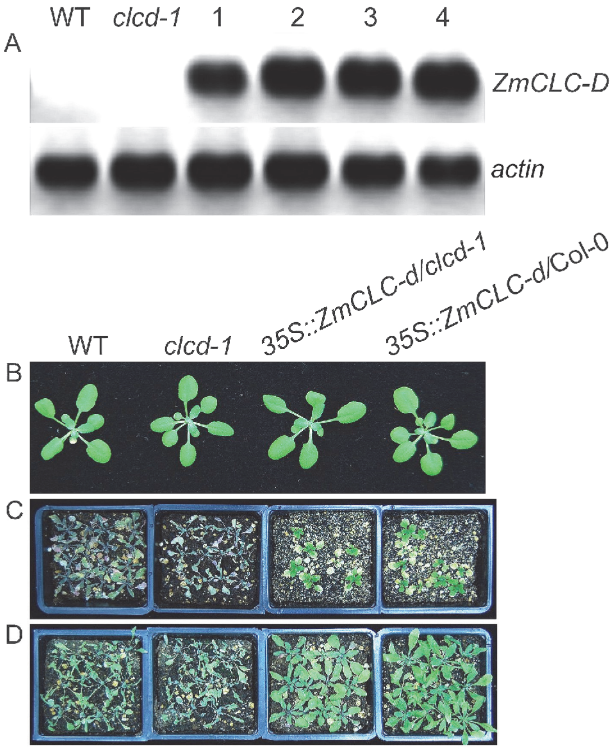

Fig. 2. The effect of *ZmCLC-d* expression on freezing, drought, and salt tolerance in transgenic *Arabidopsis* plants. *A* - The transcription of *ZmCLC-d* was analyzed by RT-PCR in WT, clcd-1, 35S::ZmCLC-d/clcd-1 (1,2), and 35S::ZmCLC-d (3,4). *B* - The phenotype of 3-week-old WT, clcd-1, and *ZmCLC-d*-overexpressing *Arabidopsis* plants at 22 °C. *C* - Four-week-old plants of the same lines as in *B* were cold treated at -10 °C for 10 h and then returned to the normal conditions for recovery. The photographs of representative seedlings were taken after 6 d of recovery. *D* - Drought tolerance was determined by growing these lines with normal watering for 2 weeks, then watering was withheld for 2 weeks, and the plants were then re-watered for 7 d before the photographs were taken.

however, most of the WT and *clcd-1* seedlings became white and did not regrow after they were moved to the normal conditions. Thus, the *ZmCLC-d*-over expressing plants showed a higher tolerance to the freezing stress.

To determine the effect of the *ZmCLC-d* overexpression on drought tolerance, 14-d-old plants grown in soil were not watered for two weeks and then watered and grown under normal conditions for 7 d (Fig. 2*D*). After resumed watering, the transgenic plants showed a stronger growth recovery than the *clcd-1* and the WT plants. Only 12.50 % of the WT plants and 15.21 % of the *clcd-1* plants survived this treatment. In contrast, 72.92 % of 35S::*ZmCLC-d/clcd-1* and 71.56 % of 35S::*ZmCLC-d* survived (Table 4) suggesting that the overexpression of *ZmCLC-d* in transgenic *Arabidopsis* resulted in a greater tolerance to the drought stress than in the wild type.

Table 4. The survival rates of transgenic plants under freezing and drought stress conditions. Total - a number of plants used in the stress assay, Survival - a number of surviving plants.

Stress	Lines	Total	Survival	[%]
Freezing	WT	87	10	11.49
	clcd-1	80	8	10.00
	35S::*ZmCLC-d/clcd-1*	91	66	72.52
	35S::*ZmCLC-d/*Col-0	101	78	77.22
Drought	WT	88	11	12.50
	clcd-1	92	14	15.21
	35S::*ZmCLC-d/clcd-1*	96	70	72.92
	35S::*ZmCLC-d/*Col-0	109	78	71.56

To test the effect of the *ZmCLC-d* overexpression on salt tolerance, we examined the effect of NaCl on the germination of *ZmCLC-d*-overexpressing seeds. No difference was observed in seed germination between the WT, *clcd-1*, and transgenic plants under the normal conditions (Table 5). At 100 mM NaCl, nearly 72.43 and 73.51 %, of the 35S::*ZmCLC-d/clcd-1* and 35S::*ZmCLC-d* seeds, respectively, germinated in comparison with 63.29 % of the WT seeds and 62.46 % of the *clcd-1* seeds. At 200 mM NaCl, the germination of the WT, *clcd-1* and *ZmCLC-d*-overexpressing seeds was completely inhibited at day 2. At day 3, the seeds of the transgenic and control plants began to germinate, but the *ZmCLC-d*-overexpressing line showed germination rates of 8.87 and 9.51 % (35S::*ZmCLC-d/clcd-1* and 35S::*ZmCLC-d*, respectively), in contrast to 2.35 % in WT and 2.15 % in *clcd-1*. Moreover, the *ZmCLC-d*-overexpressing seedlings formed longer roots than the WT and *clcd-1* plants when grown vertically under the NaCl treatment (Fig. 3*A*). When the NaCl concentration was increased to 200 mmol, the growth of the WT and *clcd-1* plants was completely inhibited, and the seedlings were not green, whereas the *ZmCLC-d*-overexpressing seedlings remained green and continued to grow. To further characterize the involvement of *ZmCLC-d* in the NaCl response, the *ZmCLC-d*-overexpressing, *clcd-1*, and WT plants were grown for 3 weeks on MS agar plates and then transferred to new MS agar plates supplemented with 50 mmol NaCl for 24 h. When grown under the normal conditions, the *ZmCLC-d*-overexpressing, *clcd-1*, and wild-type plants did not show any significant differences in the accumulation of Cl^- in roots. When treated with 50 mmol NaCl, the *clcd-1*, and wild-type plants accumulated Cl^-, but the *ZmCLC-d* overexpressing plants could efficiently exclude Cl^- under the identical stress conditions. The Cl^- content was reduced by about 15 % compared to the controls (Table 5). From these data, we can conclude that the *ZmCLC-d* overexpressing plants were more tolerant to the NaCl stress than *clcd-1* and WT.

To evaluate whether the overexpression of *ZmCLC-d* in *Arabidopsis* could increase tolerance to oxidative

Table 5. Seed germination and root chloride content in WT, *clcd-1*, and transgenic *Arabidopsis* plants under different NaCl concentrations. Means ± SD, *n* = 3. * - significant differences at $P \le 0.05$ determined by the Tukey's test.

Lines	Germination [%]					Cl- content [μmol g^{-1}(f.m.)]	
	0 mM	50 mM	100 mM	150 mM	200 mM	0 mM	50 mM
WT	95.71±0.92	83.79±0.76	63.29±0.98	34.42±0.82	2.35±0.11	18.21±1.53	90.35±4.34
clcd-1	95.04±0.61	82.64±0.81	62.46±0.56	33.91±0.46	2.15±0.15	17.47±1.76	91.73±5.06
35S::*ZmCLC-d/clcd-1*	96.11±0.87	91.24±0.69*	72.43±0.71*	52.64±0.59*	8.87±0.14*	21.74±1.94	74.52±3.72*
35S::*ZmCLC-d/*Col-0	96.83±0.93	91.48±0.89*	73.51±0.69*	54.72±0.63*	9.51±0.18*	19.94±1.88	75.28±3.83*

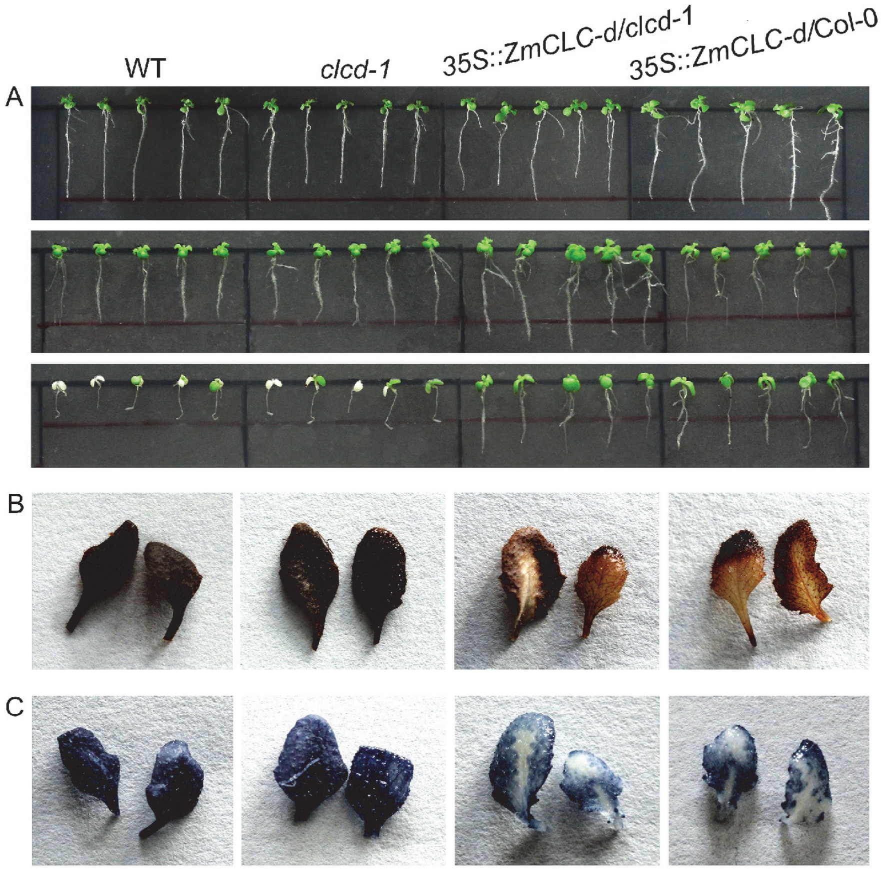

Fig. 3. The effect of *ZmCLC-d* expression on salt tolerance in transgenic *Arabidopsis* plants. *A* - Salt tolerance was determined by germinating *ZmCLC-d*-overexpressing, WT, and *clcd-1*seeds on MS agar plates and then transferring the seedlings to a new MS agar plate supplemented with different concentrations of NaCl for 7 d. *B* and *C* - The *in situ* detection of H_2O_2 and O_2^- by DAB (*B*) and NBT (*C*) staining, respectively, in leaves of WT, *clcd-1*, and transgenic seedlings.

stress, 1-month-old T_3 homozygous transgenic plants were subjected to 4 °C for 24 h. The low temperature treatment is known to cause membrane-lipid peroxidation leading to an increased MDA content. Under the normal conditions, the MDA content of the WT, *clcd-1*, and transgenic plants showed no significant differences. Following the low-temperature treatment, the MDA content in the WT and *clcd-1* plants increased up to 59.49 and 59.27 nmol g^{-1}, respectively, whereas only 46.41 and 44.96 nmol g^{-1} were observed in the 35S::*ZmCLC-d/clcd-1* and 35S::*ZmCLC-d* plants (Table 6). Furthermore, significantly higher SOD, POD, and CAT activities were detected in the *ZmCLC-d*-overexpressing plants than in the control plants in response to the cold stress (Table 6). These results demonstrate that the constitutive expression of the *ZmCLC-d* genes in *Arabidopsis* increased tolerance to oxidative stress.

Abiotic stresses induce ROS accumulation. Therefore, we evaluated the accumulation of H_2O_2 and O_2^- in the transgenic seedlings under the chilling stress. WT, *clcd-1*, and homozygous T_3 transgenic *Arabidopsis* seeds were germinated on MS medium plates for two weeks under normal conditions and were then transferred to 4 °C. After one week of the treatment, the WT and *clcd-1* plants showed greater leaf accumulations of H_2O_2 and O_2^- when compared to the *ZmCLC-d*-overexpressing lines, as indicated by DAB staining and NBT staining (Fig. 3*B*,*C*). Under the normal conditions, no obvious H_2O_2 or O_2^- was detected in either the WT or transgenic seedlings (data not shown). These results show that the *ZmCLC-d* overexpression in *Arabidopsis* effectively alleviated the ROS accumulation which is correlated with the increased antioxidant enzyme activities.

To investigate the relationship between *ZmCLC-d* and other stress marker genes, we monitored the expression of stress responsive genes identified in the appropriate pathways using RT-qPCR. Under the normal conditions (22 °C), the expressions of *CBF1*, *CBF2*, *CBF3*, *DREB2A*, and *RCI2A* in the *ZmCLC-d*-overexpressing transgenic plants were slightly higher than in the WT and *clcd-1* plants. Under the 4 °C treatment for 24 h, the tested marker genes showed a significant induction in the WT, *clcd-1*, and especially in transgenic plants (Table 7). These results indicate that the overexpression of *ZmCLC-d*, directly or indirectly, could increase the transcription of some stress marker genes.

Table 6. MDA content and CAT, POD, and SOD activities in WT, *clcd-1*, 35S::*ZmCLC-d/clcd-1*, and 35S::*ZmCLC-d* plants under control conditions and a cold stress. Means ± SD, *n* = 3, * - significant differences at $P \leq 0.05$ determined by the Tukey's test.

Parameter	[°C]	WT	*clcd-1*	35S::*ZmCLC-d/clcd-1*	35S::*ZmCLC-d/*Col-0
MDA	22	39.01±0.77	40.09±0.99	39.44±1.05	38.37±0.87
[nmol g^{-1}(f.m.)]	4	59.49±1.60	59.27±1.23	46.41±1.53	44.96±1.71
CAT	22	9.10±0.37	8.81±0.26	11.37±0.42	12.56±0.44
[μmol(H_2O_2) mg^{-1}(f.m.) min^{-1}]	4	13.48±0.42	13.33±0.58	15.30±0.75	16.88±0.76
POD	22	27.31±0.86	26.24±1.09	32.46±1.02	34.21±1.43
[U mg^{-1}(protein)]	4	33.91±1.05	33.23±1.21	42.21±1.65	43.15±1.78
SOD	22	28.22±0.73	29.13±1.01	30.31±1.12	31.36±1.15*
[U mg^{-1}(protein)]	4	35.53±1.34	34.92±1.32	45.71±1.86*	47.27±1.88*

Table 7. The expression of stress responsive genes in WT, *clcd-1*, 35S::*ZmCLC-d/clcd-1*, and 35S::*ZmCLC-d* plants under chilling stress. Means ± SD, *n* = 3, * - significant differences at $P \leq 0.05$ determined by the Tukey's test.

Parameter	[°C]	WT	*clcd-1*	35S::*ZmCLC-d/clcd-1*	35S::*ZmCLC-d/*Col-0
CBF1	22	1.00±0.14	0.93±0.18	1.67±0.26	1.57±0.21
	4	465.84±47.85	450.28±45.81	1352.89±134.81*	1465.75±141.56*
CBF2	22	1.00±0.14	1.21±0.13	0.88±0.11	1.14±0.12
	4	272.94±31.27	276.43±33.54	585.88±36.89*	591.62±35.62*
CBF3	22	1.00±0.09	1.18±0.12	1.03±0.09	1.26±0.14
	4	87.21±12.62	88.61±12.56	145.56±15.73*	158.79±17.46*
DREB2A	22	1.00±0.08	0.96±0.07	0.95±0.08	1.31±0.10
	4	83.23±13.38	80.18±12.81	218.90±22.72*	220.93±25.14*
RCI2A	22	1.00±0.12	0.89±0.07	1.69±0.12	1.58±0.11
	4	94.14±11.99	95.32±12.21	134.38±30.37*	141.28±31.28*

Discussion

In this study, we described molecular cloning chloride channel *ZmCLC-d* from maize and the improvement of stress tolerance of *Arabidopsis* transgenic plants overexpressing *ZmCLC-d*. *ZmCLC-d* is homologous to *Arabidopsis AtCLC-d*, and previous studies have demonstrated that *AtClC-d* is expressed in the trans-Golgi network (Von der Fecht-Bartenbach *et al.* 2007). Eight putative transmembrane domains were predicted in the N-terminal of *ZmCLC-d* suggesting *ZmCLC-d* may localize in the plasma membrane or membranes of organelles.

NaCl stress involves two distinct phases, a rapid osmotic phase in response to the osmotic effect of salt, followed by a slow ionic phase resulting from a toxic accumulation of Na^+ and Cl^- in the cytoplasm. Studies on NaCl tolerance have mainly focused on Na^+, as Na^+ reaches a toxic concentration in many species before Cl^- does (for review, see Munns and Tester 2008). Although Cl^- is an essential micronutrient for higher plants (White and Broadley 2001, Hänsch and Mendel 2009), it can become toxic if accumulates in the cytoplasm. Indeed, for some species, such as soybean, citrus, and grapevine, Cl^- is more toxic than Na^+ (Läuchli 1984, Storey and Walker 1999). CLCs may be involved in responses to salt stress. In soybean, GmCLC-1, which is located at the tonoplast and induced by NaCl treatment, allows better tolerance to NaCl probably by accumulating chlorides in the vacuole as it is in transgenic BY2 cells (Li *et al.* 2006). In rice, OsCLC-1, which is also located at the tonoplast, is expressed in response to NaCl treatment (Nakamura *et al.* 2006). In *Physcomitrella patens*, a member of the CLC family was identified in response to NaCl treatment by proteomic analyses (Wang *et al.* 2008). An *Arabidopsis* vacuolar anion transporter, AtCLCc, is involved in the regulation of stomatal movements and contributes to salt tolerance (Jossier *et al.* 2010). The lumen pH in the trans-Golgi network was adjusted by AtCLC-d-mediated transport of Cl^- or NO_3^-, meanwhile root growth and cell elongation were also impaired in the *AtCLC-d* loss function mutant (Von der Fecht-Bartenbach *et al.* 2007). To investigate the biological function of *ZmCLC-d*, we overexpressed it in *Arabidopsis*. We found the transgenic plants have a higher tolerance to abiotic stresses. The transgenic *Arabidopsis* seedlings expressing *ZmCLC-d* grown on the MS medium containing 200 mM NaCl for 7 d exhibited higher germination rates and longer roots than the control seedlings (Table 5). This might happen because the overexpressing *ZmCLC-d Arabidopsis* had a lower Cl^- content than the controls. Drought, heat, and chilling stresses are also accompanied by changes in ion concentrations. *ZmCLC-d* might be directly or indirectly involved in these resistance responses because we found the *Arabidopsis* seedlings overexpressing *ZmCLC-d* were more tolerant to these stresses. Our data indicate that *ZmCLC-d* played an important role in plant stress tolerance, especially in the salt stress.

In the present study, *ZmCLC-d* was also shown to be up-regulated by the H_2O_2 and ABA treatments (Table 3). Furthermore, the *ZmCLC-d*-overexpressing seeds showed a difference from the control plants during germination on MS plates that were supplemented with different concentrations (0 - 2 μM) of ABA (data not shown) suggesting that *ZmCLC-d* induced stress tolerance may be ABA-dependent, and in addition, the second messenger, H_2O_2, might participate in *ZmCLC-d* tolerance response processes.

Certain stress-induced proteins have been shown to impart stress tolerance. The overexpression of genes, such as *CBF*/*DREB1*, *OSISAP1*, and *HVA1* was able to confer stress tolerance to transgenic plants (Xin 2001, Dubouzet *et al.* 2003, Mukhopadhyay *et al.* 2004). These examples provide a target for improving the stress tolerance of crop plants and provide an opportunity to elucidate the function of previously uncharacterized genes. The *ZmCLC-d* expression in maize was induced following the exposures to the cold, drought, heat, and salt stresses (Table 3). In *Arabidopsis*, the overexpression of *ZmCLC-d* led to increased tolerance to the cold, dehydration, and salt stresses. Our data suggest that the overexpression of *ZmCLC-d* resulted in the enhanced transduction of stress-response signals. Furthermore, an elevated stress tolerance displayed by the 35S::*ZmCLC-d* plants coincided with the up-regulation of stress-responsive genes including *DREB2A*, *CBF1*, *CBF2*, *CBF3*, and *RCI2A* (Table 7). *CBF*s are thought to function in cold-responsive gene expression, whereas *DREB2s* are involved in high salinity- and drought-responsive gene expression (Liu *et al.* 1998). RCI2A protein is neither regulated by CBF/DREB1 nor involved in the CBF/DREB1-independent pathways that modulate stress signaling (Medina *et al.* 2005). The hydrophilic RCI2A protein contributes to increased stress tolerance in transgenic plants (Thomashow 1998, Hasegawa *et al.* 2000). Thus, the enhanced stress tolerance displayed by the *ZmCLC-d* transgenic plants might partially depend on changes in the expression of these genes. However, the mechanisms how *ZmCLC-d* regulated the transcription of stress induced genes are not clear.

In conclusion, we used RT-qPCR to examine the expression patterns of *ZmCLC-d* under different stress conditions. These results clearly suggest that *ZmCLC-d* was transcriptionally up-regulated in response to the cold, drought, heat, NaCl, ABA, and H_2O_2. The overexpression of *ZmCLC-d* in *Arabidopsis* enhanced its tolerance to the cold, drought, heat, and salt stresses by increasing the germination rate, root length, plant survival rate, antioxidant enzymes (CAT, POD, and SOD) activities, and reducing the accumulation of Cl^- compared with the WT plants. These results demonstrate that *ZmCLC-d* might be a positive regulator of cold, drought, heat, and salinity tolerances. The enhanced stress tolerance of the 35S::*ZmCLC-d Arabidopsis* plants reveals that ZmCLC-d was involved in stress response pathways.

Although the detailed mechanism by which ZmCLC-d is involved in stress has not yet been elucidated, the characterization of *ZmCLC-d* function provides new insights into stress pathways. This report broadens knowledge of the function of *CLC-d* in plants and provides beneficial information regarding molecular breeding that could lead to improved stress tolerance of agricultural crops.

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