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Constitutive expression of abiotic stress-inducible hot pepper *CaXTH3*, which encodes a xyloglucan endotransglucosylase/hydrolase homolog, improves drought and salt tolerance in transgenic *Arabidopsis* plants☆

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Abstract Xyloglucan endotransglucosylase/hydrolase (XTH) has been recognized as a cell wall-modifying enzyme, participating in the diverse physiological roles. From water-stressed hot pepper plants, we isolated three different cDNA clones (pCaXTH1, pCaXTH2, and pCaXTH3) that encode XTH homologs. RT-PCR analysis showed that three *CaXTH* mRNAs were concomitantly induced by a broad spectrum of abiotic stresses, including drought, high salinity and cold temperature, and in response to stress hormone ethylene, suggesting their role in the early events in the abiotic-related defense response. Transgenic *Arabidopsis* plants that constitutively expressed the *CaXTH3* gene under the control of the CaMV 35S promoter exhibited abnormal leaf morphology; the transgenic leaves showed variable degrees of twisting and bending along the edges, resulting in a severely wrinkled leaf shape. Microscopic analysis showed that *35S-CaXTH3* leaves had increased numbers of small-sized cells, resulting in disordered, highly populated mesophyll cells in each dorsoventral layer, and appeared to contain a limited amount of starch. In addition, the *35S-CaXTH3* transgenic plants displayed markedly improved tolerance to severe water deficit, and to lesser extent to high salinity in comparison with the wild-type plants. These results indicate that CaXTH3 is functional in heterologous *Arabidopsis* cells, thereby effectively altering cell growth and also the response to abiotic stresses. Although the physiological function of CaXTHs is not yet clear, there are several possibilities for their involvement in a subset of physiological responses to counteract dehydration and high salinity stresses in transgenic *Arabidopsis* plants.

Keywords: Abiotic stress; Cell wall; Coordinated gene expression; Hot pepper; Transgenic *Arabidopsis*; Xyloglucan endotransglucosylase/hydrolase

1. Introduction

During their entire life cycle, higher plants routinely encounter various environmental stresses. Drought, high salinity, heavy metals, and extreme temperatures are common abiotic stresses, which impair the growth and development of soil plants. [1,2]. The molecular and cellular processes underlying the acclimation of higher plants to abiotic stresses have attracted much interest, as environmental stress conditions result in the seriously loss of crop production in many parts of the world [3,4]. Using molecular and genetic approaches, a large and increasing number of genes induced by abiotic stresses have been identified recently [3,5–9]. In addition, signal transduction pathways and cellular events that occur under such unfavorable growth conditions have been widely documented [10–12]. Nevertheless, our understanding about the cellular functions of stress-inducible genes with regard to either stress tolerance or sensitivity in crop plants is still rudimentary. Thus, it is crucial to study the functions of stress-related genes to understand the molecular mechanisms of stress tolerance in crop plants.

Hot pepper (*Capsicum annuum* L.) is a solanaceous species that is closely related to tobacco. It is one of the most economically important crops and cultivated widely in East Asia for its hot-tasting fruits. We are interested in elucidating the adaptive response of hot pepper plant in response to abiotic stresses, such as water deficit. Previously, we have isolated and characterized a broad spectrum of cDNAs from hot pepper plants whose expression is enhanced rapidly in response to dehydration [13–15]. Among the identified cDNAs, pCa-DI1, pCa-DI2 and pCaDI3 encode partial proteins that are homologous to xyloglucan endotransglucosylase/hydrolase (XTH) enzymes [14]. In the present study, we have isolated three different full-length *Ca-DI* cDNA clones, renamed *CaXTHs* for *C. annuum* XTH homologs, and analyzed the detailed expression pattern of their mRNAs in response to various abiotic stresses and plant hormone. We also present results indicating that over-expression of *CaXTH3* markedly improves tolerance to drought and also confers increased tolerance to high salinity in transgenic *Arabidopsis* plants.

2. Materials and methods

2.1. Plant materials and application of various stresses

Dry hot pepper seeds (*C. annuum* cv. Pukang) were soaked once with 70% ethanol and then rinsed extensively with sterilized water. Seedlings were grown in a mixture of soil and vermiculite or on MS medium

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Abbreviations: RT-PCR, reverse transcriptase-polymerase chain reaction; TEM, transmission electron microscopy; XTH, xyloglucan endotransglucosylase/hydrolase

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containing 1% sucrose, B5 vitamin (12 mg/l), and 0.8% agar (pH 5.8) in a 25 °C-growth chamber with a 16-h-light/8-h-dark photoperiod. Hot pepper plants were subjected to various abiotic stresses, such as drought, high salinity and clod as described [14]. For ethylene treatment, intact plants were enclosed for various periods in 3-liter jars containing air or air plus 50 μl/l of ethylene as described previously [16].

2.2. RNA isolation and RNA gel blot analysis

Total RNAs of hot pepper and transgenic *Arabidopsis* plants were obtained as described by Mang et al. [17] and Lee et al. [18], respectively. The total RNAs were precipitated overnight at 4 °C by the addition of 0.3 vol. 10 M LiCl and then precipitated in ethanol. The RNA gel blots were hybridized to various ^{32}P-labeled cDNA probes for *CaXTH1, CaRCI* and *CaPINII* under normal stringent hybridization and washing conditions. The blot was washed and visualized by autoradiography at −80 °C using Kodak XAR-5 film and an intensifying screen. Hybridization signals were quantified with a PhosphorImager (Fuji).

2.3. Reverse transcriptase-polymerase chain reaction

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in a total volume of 25 μl containing 1 μl of the first strand cDNA reaction products, 1 μM gene-specific primers (Table 1), 10 mM Tris (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM deoxynucleotides and 2.5 units of Taq polymerase (Promega, Madison, WI, USA). Twenty-five thermal cycles were carried out, each consisting of 45 s at 95 °C, 1 min at 60 °C, and 90 s at 72 °C in an automatic thermal cycler (Perkin–Elmer/Cetus, Norwalk, CT, USA). PCR products were separated on a 1% agarose gel and then visualized under UV light.

2.4. Generation of the 35S-CaXTH3 construct and transformation of Arabidopsis

The full-length pCaXTH3 cDNA was inserted into the corresponding sites of the binary vector pBI121. The fusion gene construct was transferred to *Agrobacterium tumefaciens* strain AGL1 by electroporation, as described previously [19]. *Arabidopsis* transformation was accomplished by the floral dip method [20]. The seeds collected from the plants were selected on a 0.5× MS plate containing 25 mg/l kanamycin to obtain independent transgenic lines. The presence of the transgene was confirmed by PCR and genomic Southern blot analysis. Homozygous T4 lines were obtained by further self-crossing according to Joo et al. [19] and used in this study.

2.5. Histochemical analysis

Mature leaves of four-week-old wild-type and transgenic *Arabidopsis* plants were harvested at 10:00 AM of the 16-h-light/8-h-dark photoperiod for histochemical analysis and iodine staining experiment. Light microscopy and transmission electron microscopy (TEM) on transgenic *Arabidopsis* leaves were performed as described [21].

2.6. Measurement of root length of wild-type and transgenic Arabidopsis seedlings

Wild-type and transgenic *Arabidopsis* seeds were surface-sterilized and plated on 0.8% agar plates (select agar; Life Technology, Rockville, MD, USA) containing 0.5× MS salts, 0.5 mM MES, pH 5.7, 1% sucrose, and 1× vitamin B5. To test root growth, vertically oriented agar plates containing 4-day-old seedlings were incubated at 22 °C in a 16-h-light/8-h-dark cycle for 6 days in the absence or presence of NaCl (50–100 mM). During incubation, the advancing root tips were recorded using a marker pen on the outside of the plates, and the image of the marked plate was scanned using ScionImage software (Scion Corp., Frederic, MD, USA).

Table 1
Primer sequences used in the RT-PCR analysis

Gene name	Accession Number	Primer sequence
CaXTH1	DQ439860	5′-ATCCCATTTCATCTTCAAATTAAAGC-3′ 5′-GGGGAAATGATTTATTGTTATTTCG-3′
CaXTH2	DQ439861	5′-CTATGCCCGGCAGCTTGGGCTGAA-3′ 5′-GACAACATTAGTAAACTCAATCC-3′
CaXTH3	DQ439862	5′-GTGGGCTGAGAATTTTTACCAAGAT-3′ 5′-GGCAAGAAAACCATTCATTGTTATTTTCTA-3′
Actin	AY572427	5′-ACTCTTAATCAATCCCTCCACC-3′ 5′-CTGTATGACTGACACCATCACC-3′

2.7. Germination assay

For germination assay, wild-type and transgenic seeds collected at the same time were used. Germination ratio was monitored in the absence or presence of various concentrations of NaCl (50–100 mM) or ABA (0.1–1 μM).

2.8. De-colorization and staining for starch with an iodine solution

Mature leaves from wild-type and transgenic plants were decolorized and stained for starch with an iodine solution as described by Zeeman et al. [22].

3. Results

3.1. Isolation and identification of the full-length CaXTH cDNAs

With the aid of subtractive hybridization and differential display PCR analyses, we previously isolated a broad spectrum of partial cDNA clones from hot pepper seedlings, which were rapidly induced by water deficit [13–15]. Among the identified clones, three different cDNA clones, pCa-DI1, pCaDI2, and pCaDI3, encoded partial polypeptides homologous to XTHs in various plant species [14]. Southern blot analysis on hot pepper genomic DNA showed that there exist at least 5–6 bands that hybridize differentially with the pCa-DI1 probe [14], which is consistent with previous results that XTH is encoded by a closely related gene family in tomato [23,24], *Arabidopsis* [25] and rice [26,27]. Similar patterns of hybridization was also observed with pCa-DI2 and pCa-DI3 probes, respectively [14]. Thus, it is most likely that homologous Ca-DI1, Ca-DI2 and Ca-DI3 genes are not cloning artifact, but they are indeed a gene family encoding the hot pepper XTH enzymes. Thus, these clones were renamed *CaXTH*, for *C. annuum* XTH homologs, and used to isolate full-length pCaXTH cDNA clones. The partial cDNA fragments, pCa-DI1, pCaDI2, and CaDI3, were radioactively labeled, mixed together, and then used as probes to screen the lambda uni-Zap II cDNA library constructed from water-stressed leaves of hot pepper plants. Numerous putative *CaXTH* cDNA clones were isolated. Subsequent restriction enzyme mapping and DNA sequencing analysis revealed that these clones represented three distinct CaXTH sequences. Fig. 1A shows the restriction map analysis of pCaXTH1, pCaXTH2 and pCaXTH3, which contain the longest insert among each homology class. All three clones were completely sequenced. The pCaXTH1 clone (GenBank Accession No. DQ439860) is 1142 bp long and encodes 287 amino-acids, pCaXTH2 (GenBank Accession No. DQ439861) is 1106;bp long and encodes 288 amino-acids, and pCaXTH3 (GenBank Accession No. DQ439862) is 1088 bp in length and encodes 287 amino-acids (Fig. 1A and B). The deduced amino-acid sequence identity between *CaXTH1* and *CaXTH2* is 94%, between *CaXTH1* and *CaXTH3* is 96%, and between *CaXTH2* and *CaXTH3* is 94%. These results indicate that the hot pepper *CaXTH* gene family is highly conserved, with *CaXTH1* and *CaXTH3* being the more homologous members of this gene family (Fig. 1B).

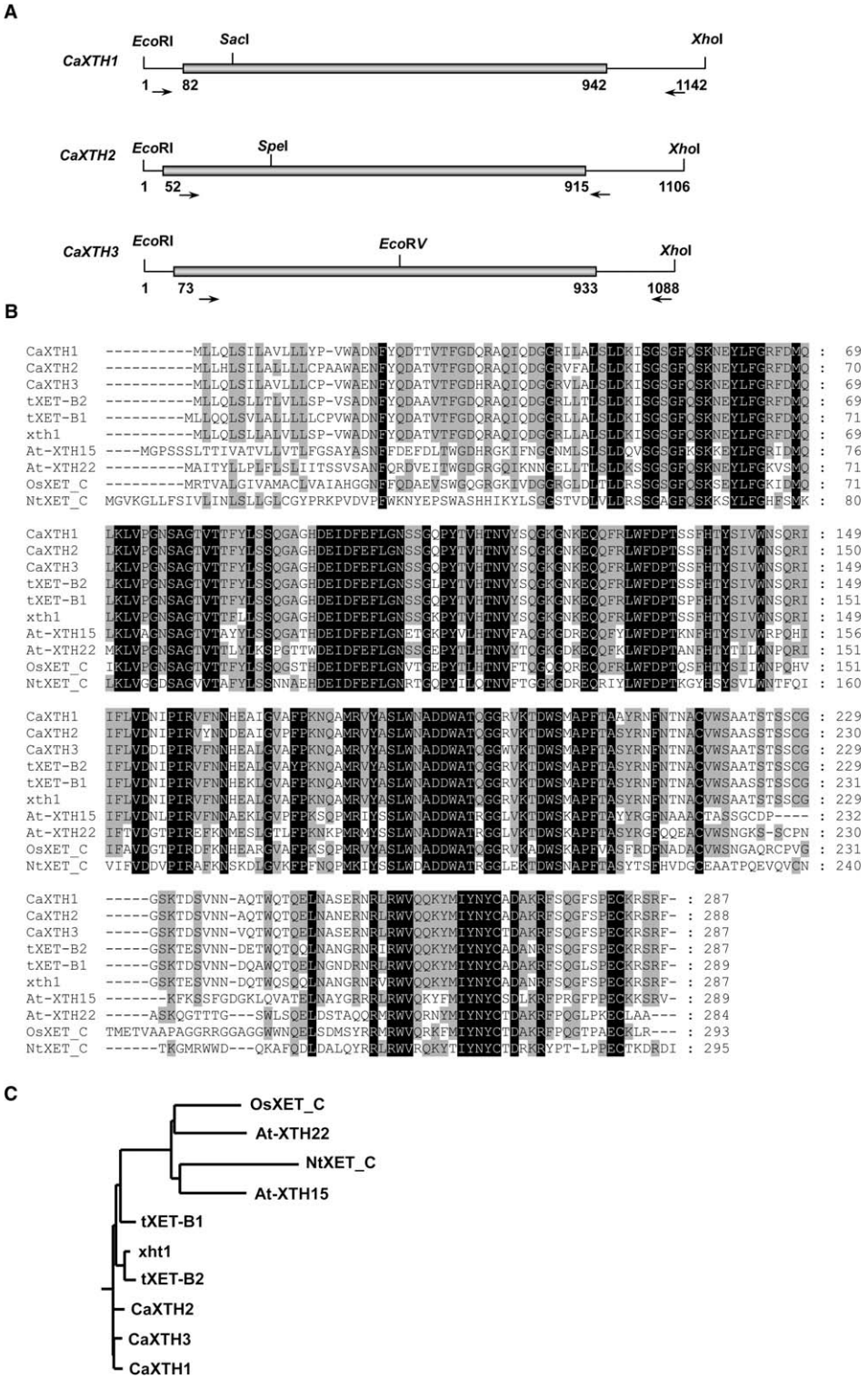

Fig. 1. Sequence analysis of hot pepper CaXTH homologs. (A) Restriction enzyme map analysis of the hot pepper *CaXTH* cDNA clones. Solid bar depicts the coding region. Solid lines represent 5′- and 3′-untranslated regions. The positions of gene-specific primers used for RT-PCR are indicated. The sequences of pCaXTH1, pCaXTH2, and pCaXTH3 have been deposited in the GenBank database under Accession Nos. DQ439860, DQ439861 and DQ439862, respectively. (B) Comparison of the derived amino-acid sequence of hot pepper CaXTH homologs with those of the tomato (tXET-B1 and tXET-B2) [23,24], potato (xth1) (Genbank Accession No. CAJ77496), *Arabidopsis* (At-XTH15 and At-XTH22) [25], rice (OsXET_C) [26,27] and tobacco (NtXET_C) [28]. Amino-acid residues that are conserved in at least seven of the ten sequences are shaded, whereas amino-acids that are identical in all ten proteins are shown in black. Dashes show gaps in the amino-acid sequences that were introduced to optimize alignment. (C) Phylogenetic relationship of XTHs from various plant species.

In addition, *CaXTH* genes exhibit a considerable degree of sequence identity when compared with *XTH* genes from tomato (tXET-B1 and tXET-B2, 90–92%) [23,24], *Arabidopsis* (At-XTH15 and At-XTH22, 60–61%) [25], potato (xth1, 92–93%) (Genbank Accession No. CAJ77496), tobacco (NtXET_C, 46–47%) [28] and monocot rice (OsXET_C, 61–62%) [26,27] plants (Fig. 1B and C).

3.2. The CaXTH genes are induced concomitantly in response to drought, high salinity, cold temperature and ethylene in hot pepper plants

From the results described above, it appears that three homologous pCaXTH cDNAs are expressed in water-stressed leaves of hot pepper plants. This raises the possibility that the *CaXTH* gene family is expressed coordinately in response to abiotic stresses. To investigate this possibility, their mRNA accumulation profiles were monitored under various abiotic stress conditions. As a first step, two-week-old light-grown hot pepper plants harvested from agar plates were dehydrated on Whatman 3MM filter paper at room temperature and approximately 60% humidity under dim light. The degree of water stress was determined by the decrease in the fresh weight of the plants. Total RNAs were then prepared from the treated tissues after increasing exposure times, and the changes in steady-state level of mRNAs were monitored by RNA gel blot analysis using ^{32}P-labeled pCaXTH1 as a probe under normal stringent conditions, or by RT-PCR using gene-specific primers (Fig. 2A and Table 1). The results of RT-PCR show that the low, basal levels of the transcripts (~1.2 kb) corresponding to *CaXTH1*, *CaXTH2* and *CaXTH3*, respectively, begin to elevate concomitantly in response to a 5% water loss (Fig. 2B). Although this increase was seen in both leaf and root tissues, the induction kinetics of the transcripts were distinct in different plants parts. The expression of *CaXTH* mRNAs attained a maximal level at 10–15% water loss, and thereafter decreased in leaf tissue, whereas a high level of the *CaXTH* transcripts was still evident after more severe water loss (20–30%) in root tissue (Fig. 2B). Next, the expression profiles of *CaXTHs* were investigated under salt stress. Rapid induction of all three *CaXTH* transcripts was clearly detected after a 10-min treatment of the root tissue with 200 mM NaCl (Fig. 2C). This marked increase in mRNA level was continuously maintained for at least 1 h, and subsequently declined. The *Ca-RCI* gene, an *Arabidopsis RCI2A* homolog, was included in the RNA expression experiments to act as a positive control for salt stress [14]. Its mRNA began to accumulate in root tissue at 1 h, attained a maximal level at 3 h, and thereafter declined, strongly suggesting that induction of *CaXTHs* was not an

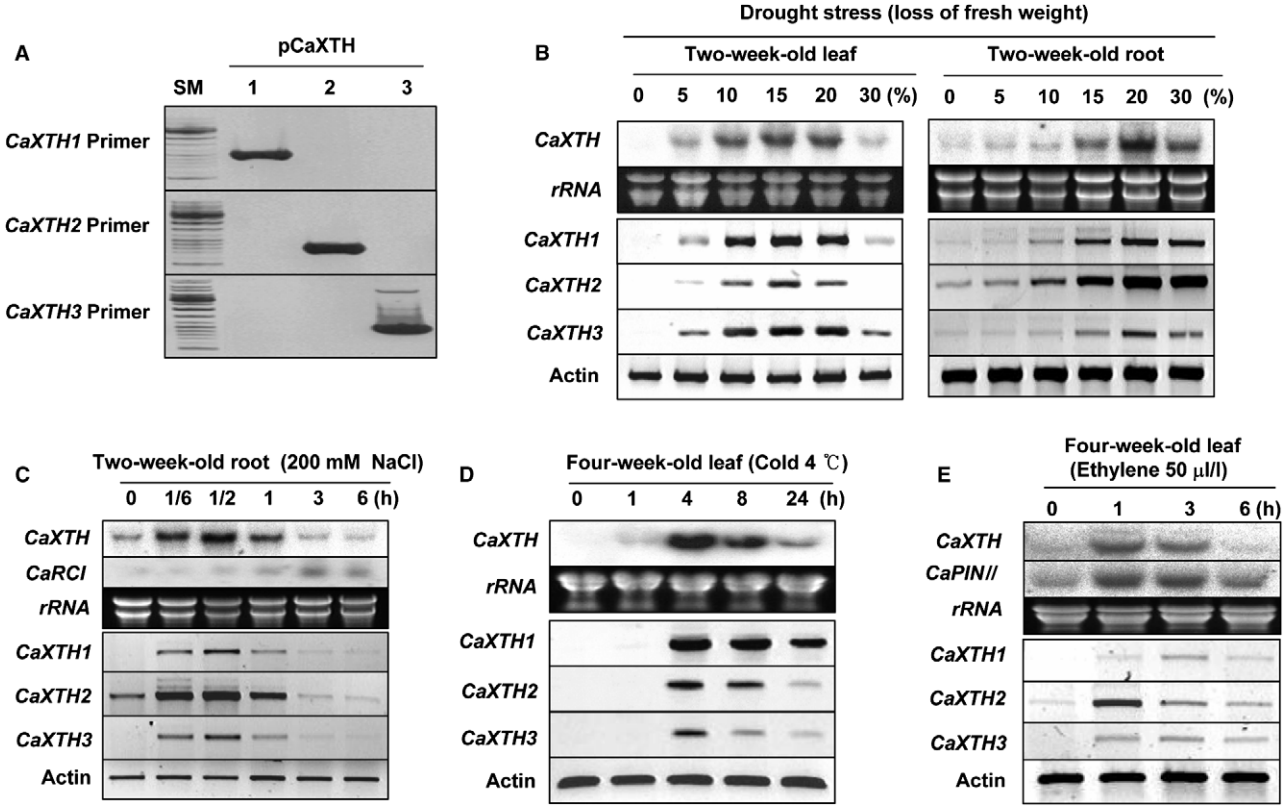

Fig. 2. Induction kinetics of *CaXTHs* in response to conditions of environmental stress in hot pepper plants. (A) Specificity of each gene-specific primer. Three different gene-specific primers for *CaXTH1*, *CaXTH2* and *CaXTH3* were constructed as shown in Fig. 1A and Table 1, and their specificity was tested for PCR analysis. (B–E) Light-grown two- or four-week-old hot pepper plants were subjected to drought (0–30% loss of fresh weight) (B), NaCl (200 mM) (C), cold temperature (4 °C) (D), or plant hormone ethylene (50 μl/l) (E). The treated tissues were harvested at the indicated time points and total RNAs were isolated. Total RNAs (20 μg) were separated by electrophoresis on a 1%-formaldehyde–agarose gel and blotted to a Hybond-N nylon membrane. To ensure equal loading of the RNA, the gel was stained with ethidium bromide after electrophoresis. To confirm complete transfer of RNA to the membrane filter, both gel and membrane were viewed under UV light after transfer. The filter was hybridized with ^{32}P-labeled pCaXTH1, pCaRCI or pCaPINII under normal stringent hybridization and washing conditions. To monitor the individual induction pattern of the *CaXTH* genes, three different gene-specific primers for *CaXTH1*, *CaXTH2* and *CaXTH3* were used for RT-PCR.

experimental artifact but specific to NaCl treatment (Fig. 2C). Likewise, cold temperature (4 °C) also coordinately enhanced *CaXTH* gene expressions in the light-grown, 7-day-old seedlings during 4–24 h treatment (Fig. 2D). As a next experiment, intact hot pepper seedlings were enclosed in 3-liter jars containing 50 μl/l of ethylene. As indicated in Fig. 2E, ethylene effectively upregulated three *CaXTHs* in leaf tissue. The *Ca-PI-NII* gene, a proteinase inhibitor II homolog, was also rapidly induced upon ethylene treatment. Thus, these results demonstrate that the *CaXTH* gene family is concomitantly activated in response to a broad spectrum of abiotic stresses in hot pepper plants, indicating their roles in the early events in the abiotic-related defense response.

3.3. Phenotypic analysis of CaXTH3 over-expressing transgenic Arabidopsis plants

To address the cellular function of the *CaXTH* gene family, we attempted to establish transgenic hot pepper plants that constitutively expressed the *CaXTH* genes. Unfortunately, this approach turned out to be unsuccessful due to the technical difficulties. Transformation and regeneration yield was extremely low so that we could not obtain the enough independent transgenic lines. Therefore, we constructed transgenic *Arabidopsis* plants that over-expressed *CaXTH3* under the control of the CaMV 35S constitutive promoter. Numerous independent T4 transgenic lines that exhibited enhanced levels of the *CaXTH3* transcript under normal growth conditions were chosen for further analysis (Fig. 3A). Fig. 3B shows the morphological comparison of *35S-CaXTH3* and wild-type plants. During our search for phenotypic differences, we observed that

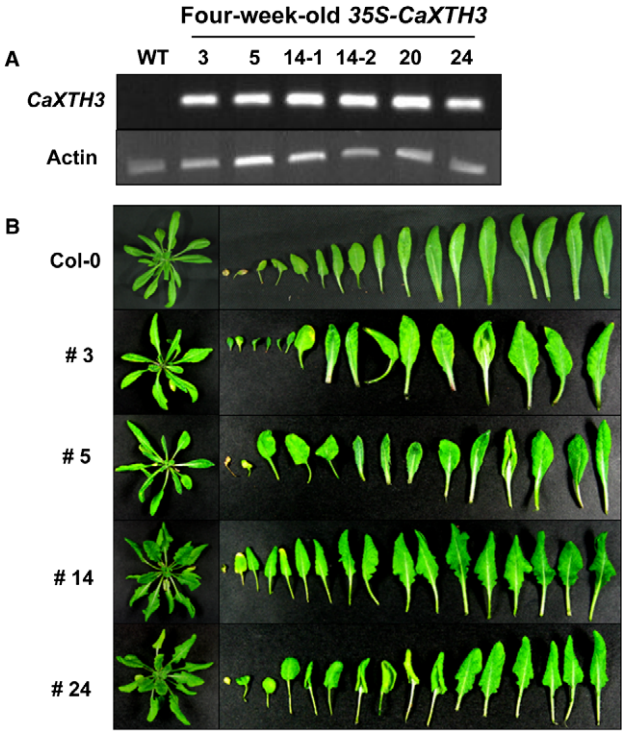

Fig. 3. Molecular characterization and phenotype of *CaXTH3* over-expressing transgenic *Arabidopsis* plants. (A) RT-PCR analysis of the four-week-old wild-type and independent *35S-CaXTH3* transgenic lines. (B) Morphological comparisons of the four-week-old wild-type and *CaXTH3* over-expressing lines under normal growth conditions.

the majority of independent *35S-CaXTH3* plants displayed abnormal leaf morphology; the transgenic leaves showed variable degrees of twisting and bending along the edges, resulting in a severely wrinkled leaf shape (Fig. 3B). As shown in Fig. 3B, the aberrant morphology generally appeared in early development of leaves.

The detailed cellular phenotype was further investigated by comparing transverse leaf sections of wild-type and *35S-CaXTH3 Arabidopsis* plants. The control leaves had a typical leaf structure of dicotyledonous plants with distinct adaxial and abaxial epidermal layers (Fig. 4A). In the *35S-CaXTH3* (transgenic line #14) leaves, a dorsoventral organization was also maintained. However, the transgenic leaves had increased numbers of small-sized cells, resulting in disordered, highly populated cells in each layer, as compared with wild-type leaves (Fig. 4A). This phenotype was also observed in transgenic lines #5 and #24 (data not shown). The leaf cells were further investigated by TEM. As shown in Fig. 4B-2, the general structure of cell wall in the *35S-CaXTH3* mesophyll cells appeared to be comparable to that in control cells. A unique phenotype specific for *CaXTH3* over-expressing cells was a marked decrease in starch formation. Fig. 4B-4 revealed that starch granule was almost undetectable in the chloroplasts of *35S-CaXTH3* leaf cells, whereas control cells exhibited well-organized starch granules in stroma region of chloroplast. To confirm this phenotype, leaves from control and transgenic plants were decolorized, and stained for starch with an iodine solution. The results in Fig. 4C show that the starch present in the leaves of wild-type plants stained dark greenish brown. On the other hand, there was background degree of iodine staining in the mature leaves from two independent *35S-CaXTH* lines (#14 and #24) (Fig. 4C), indicating that *CaXTH3* over-expressing plants accumulate a limited amount of starch in their leaves.

3.4. Over-expression of CaXTH3 improves drought and salt tolerance in Arabidopsis

The aforementioned results concerning the RNA expression profile led us to hypothesize that the hot pepper CaXTHs might function in the defense mechanism against abiotic stresses. Therefore, the effects of *CaXTH3* over-expression on the response of *Arabidopsis* to water- and salt-stresses were examined. First, the root growth assay was performed with the transgenic and wild-type seedlings that had been incubated with 50–100 mM NaCl. As illustrated in Fig. 5A, significant differences were observed between the *35S-CaXTH3* and wild-type plants after being exposed to NaCl treatment. Whereas the elongation of control roots was reduced about 40% in the presence of 100 mM NaCl, growth of the transgenic roots was less impaired (28–29% reduction) by this high salinity, indicating increased tolerance to salt stress (Fig. 5A). Under our experimental conditions, germination ratio of the wild-type plants was reduced 15% by 50 mM NaCl and 25% by 100 mM NaCl (Fig. 5B). On the other hand, the germination efficiency of the *35S-CaXTH3* plants was less affected (10–14% reduction) by 100 mM NaCl. Thus, both the germination (Fig. 5B) and post-germination (Fig. 5A) growth of *35S-CaXTH3* transgenic plants are more tolerant to high salinity than wild-type plants. Furthermore, the *CaXTH3* over-expressing plants exhibited increased resistance to exogenously applied ABA (0.1–1 μM) in germination compared with the wild-type *Arabidopsis* plants (Fig. 5C).

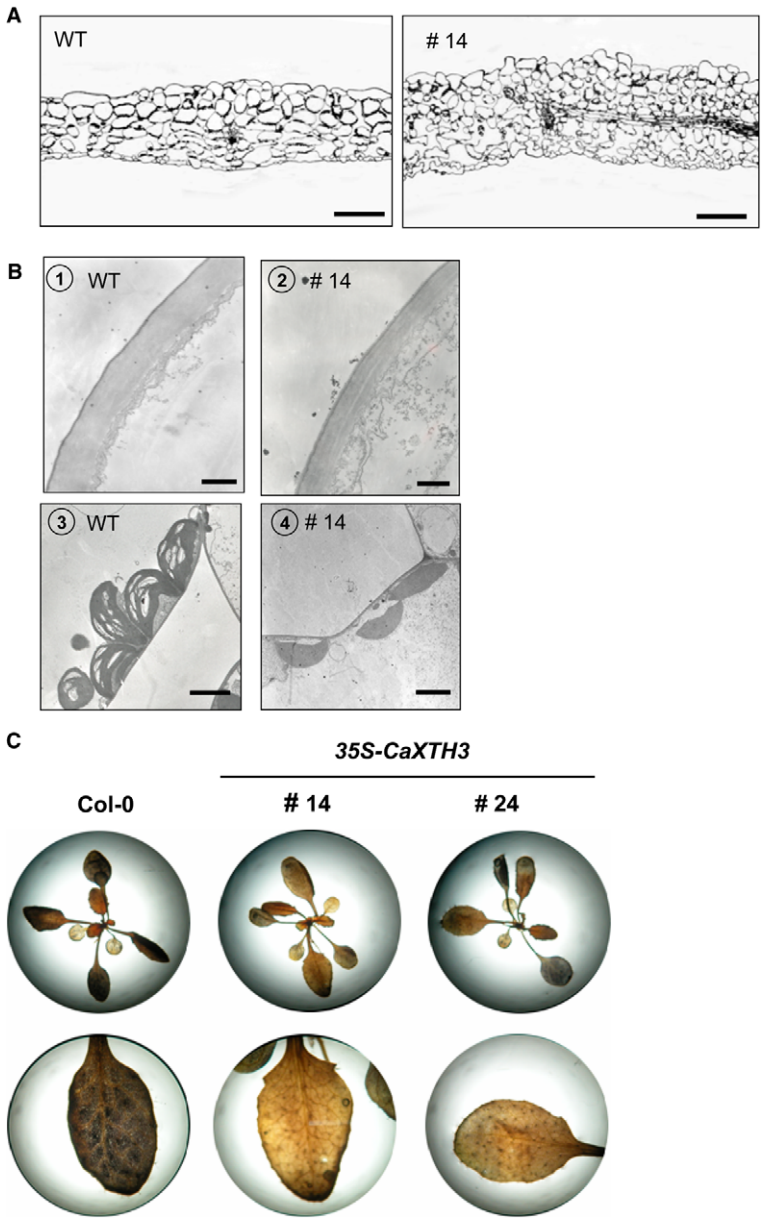

Fig. 4. Cellular phenotypes of wild-type and *35S-CaXTH3* leaves. (A) Light micrographs of leaf transverse sections of four-week-old wild-type and *35S-CaXTH3* plant (line #14). Scale bars = 100 μm. (B) Transmission electron micrographs of the mesophyll cells from four-week-old wild-type (panels 1 and 3, scale bars = 1 μm) and *35S-CaXTH3* plants (line #14) (panels 2 and 4, scale bars = 5 μm). (C) Leaves of wild-type and *35S-CaXTH3* plants (lines #14 and #24) stained for starch with iodine. Plants at the end of a 16 h photoperiod were decolorized in hot 80% (v/v) ethanol, and stained with an iodine solution.

As a next experiment, we compared the capacity of the wild-type and *35S-CaXTH3* plants to respond to dehydration. Dehydration sensitivity was scored as the capacity of plants to resume growth after water stress, when returned to normal conditions. Four-week-old *Arabidopsis* plants were grown in pots. When the soil was allowed to dry by withholding water for 15 days, wild-type plants displayed wilting (Fig. 6). After re-watering for 5 days, the majority of control plant was unable to recover and eventually died. Under these experimental conditions, however, most of the *CaXTH3* over-expressing lines appeared to be healthy before and after re-watering; they successively survived and continued to grow, as opposed to wild-type plants, under severe water stress (Fig. 6). These results, in conjunction with the data in Fig. 5, indicate that the *35S-CaXTH3* transgenic plants were highly tolerant to severe water deficit, and to lesser extent to high salinity (50–100 mM). Overall, we interpreted these results to suggest that *CaXTH3* might be involved in the control of plant responses to counteract the unfavorable growth conditions.

4. Discussion

The molecular and cellular processes underlying the acclimation of hot pepper to abiotic stresses have attracted much interest, as it is an economically important crop and its re-

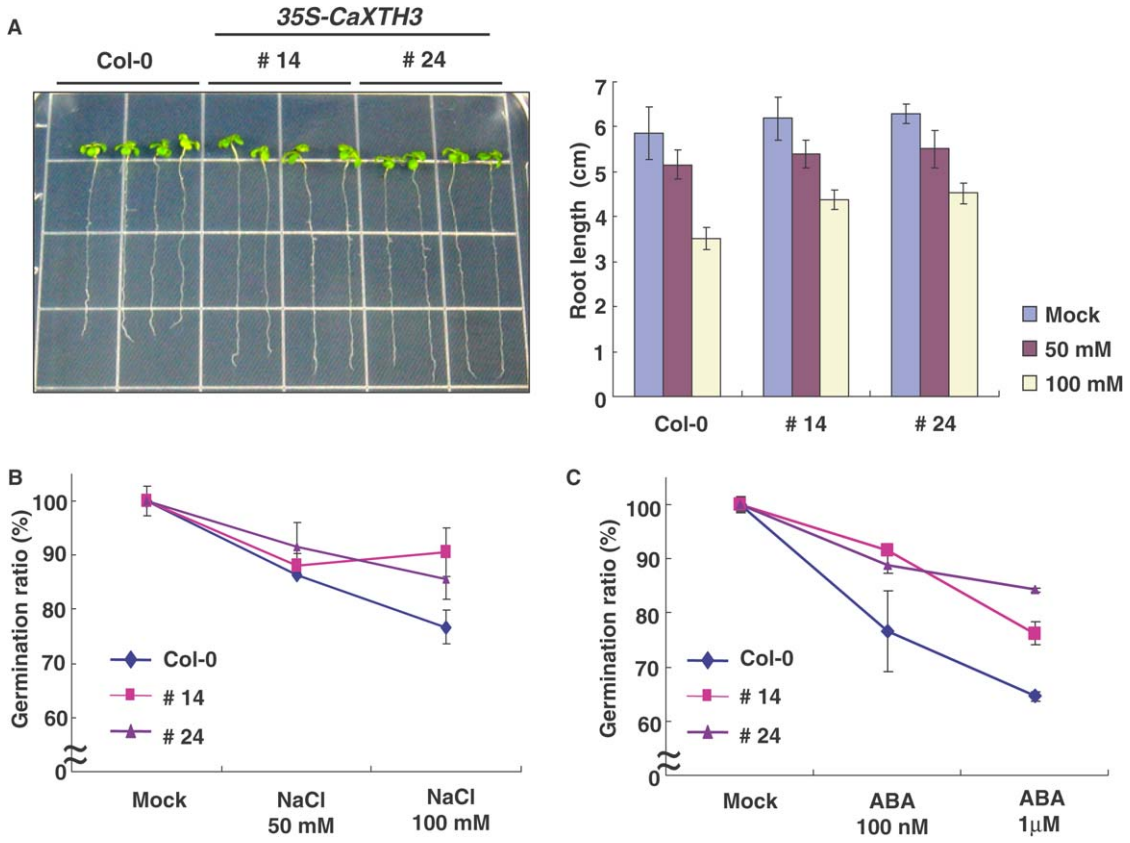

Fig. 5. Increased tolerance of *35S-CaXTH3* transgenic lines to salt stress. (A) Four-day-old light-grown wild-type and transgenic seedlings (lines #14 and #24) were subjected to 100 mM NaCl, and the growth patterns of roots were monitored after 6 days. The values are means ± S.D. (n = 30). (B) Germination ratio of the wild-type and *35S-CaXTH3* transgenic lines (lines #14 and #24) in the absence or presence of NaCl (50–100 mM). The values are means ± S.D. ($n = 30$). (C) Germination ratio of wild-type and *35S-CaXTH3* transgenic lines (lines #14 and #24) in the absence or presence of ABA (0.1–1 μM). The values are means ± S.D. ($n = 30$).

sponse to adverse environmental factors is not well understood compared to other crop plants. From water-stressed hot pepper plants, we isolated three homologous *CaXTH* genes that encode xyloglucan endotransglucosylase/hydrolase homologs identified from various plant species (Fig. 1). RT-PCR studies with gene-specific primers showed that, in hot pepper seedlings, three different *CaXTH* mRNAs were concomitantly inducible in response to diverse environmental stresses, including dehydration, high salinity, and cold temperature (Fig. 2). It was worth noting that *CaXTHs* were also rapidly activated (within 1 h) by stress hormone ethylene. Thus, it would be reasonable to consider that, in hot pepper, the CaXTH isoenzymes may be functional in early event in the abiotic-related defense response to deal with the effective plant adaptation process.

XTHs, along with pectinase and expansin, have been recognized as wall-modifying proteins, participating in the multiple physiological roles [29–32]. A wealth of evidence has been documented that high XTH enzyme activity and its mRNA level were closely correlated with the elongation zone of stems and roots and in ripening fruits in various plant species [23,26,27,33–38]. Analysis of tobacco plants transformed with an antisense construction of *NtXET-1* suggested that the reduction in *Nt-XET-1* expression might be coupled with strengthening of cell walls [28]. Most recently, Wu et al. [39] reported that XTH activity was decreased in the elongation region of soybean seedlings at low water potential. All of these results are consistent with the notion that XTHs function in regulating cell wall loosening and extensibility [30]. On the other hand, it was proposed that XTHs have a role not only in wall loosening but also in wall biogenesis and reinforcement [29,40]. Recent study by Bourquin et al. [41] provided the evidence that XTHs were actively involved in creating and reinforcing the connection between the primary and secondary wall layers of vascular tissues in poplar stems. Moreover, Arabidopsis *At-XTH22* (*TCH4*) has been known to be induced by environmental stimuli, including touch, heat shock and cold stress [25,42]. Overall, it is likely that XTHs have roles in both cell expansion and the mechanical stress response to undergo wall reinforcement.

To explore possible cellular functions of abiotic stress-inducible hot pepper CaXTHs, the *35S-CaXTH3* transgenic *Arabidopsis* plants were generated. Several lines of evidence indicate that CaXTH3 is functionally relevant in the heterologous *Arabidopsis* cells. Ectopic expression of *CaXTH3* caused a clearly distinct phenotype in comparison with the control plants, such as altered leaf morphology (Figs. 3 and 4) and, more importantly, improved tolerance to drought and salt stresses (Figs. 5 and 6). A closer inspection revealed that the transgenic leaves had highly populated small-sized cells and contained little amount of starch content relative to control leaves (Fig. 4). We consider the possibility that transgenic plants may need more metabolic energy to actively produce increased numbers of cells so that starch reservoir may not be accumulated in

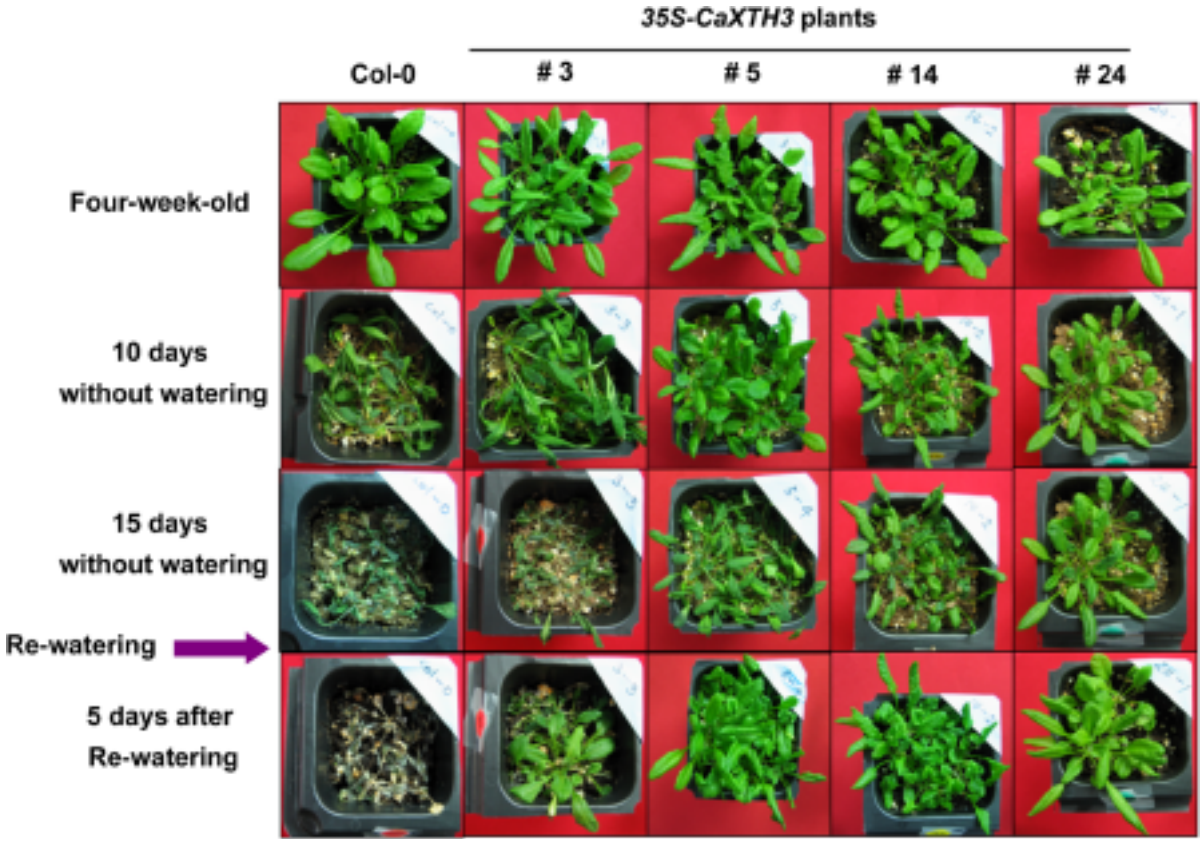

Fig. 6. Increased tolerance of *35S-CaXTH3* transgenic lines to water stress. Wild-type and transgenic *Arabidopsis* plants (lines #3, #5, #14 and #24) were grown in pots for four weeks under normal growth conditions. Thereafter, water was withheld for 15 days, followed by re-watering for 5 days. Dehydration tolerance was assayed as the capability of plants to resume growth when returned to normal conditions following water stress.

leaves. Taken together, it seems likely that the CaXTH3 is actively involved in the cellular processes, thereby effectively altering a subset of cell growth control factors, as well as the response to abiotic stresses in transgenic lines.

The critical question that remains to be unraveled is the physiological link between the functional relevance of CaXTHs and increased tolerance to drought and salt stresses in transgenic *Arabidopsis* plants. We are tempted to assume that CaXTH3 may be involved in the cell wall remodeling to strengthen the wall layers, and hence it participates in the protection of mesophyll cells against water deficit. In addition, constitutive presence of CaXTH3 enzyme may enhance cell wall biogenesis, which in turn, results in the formation of numerous small-sized cells in leaves (Fig. 4). At this moment, however, we do not know whether or not the increased number and surface area of leaf cells are associated with the tolerance to abiotic stresses in *35S-CaXTH3* plants. In this regard, it is of immense importance to analyze detailed structure and chemical components of cell wall in *35S-CaXTH3* transgenic plants. We are now attempting to establish hot pepper plants that suppress the *CaXTH* genes by virus-induced gene silencing (VIGS). VIGS will be an alternative experimental method to overcome technical problem in constructing transgenic hot pepper plants. This approach would help to further elucidate the mode of action of CaXTHs in plants. In conclusion, the data presented in this report suggest that hot pepper CaXTHs play roles in the process in response to a broad spectrum of abiotic stresses. Further experiments are now required to more precisely define the biochemical and physiological functions of CaXTHs in response to adverse environmental factors in hot pepper plants.

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