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Capsicum annuum homeobox 1 (CaHB1) is a nuclear factor that has roles in plant development, salt tolerance, and pathogen defense

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ABSTRACT

Homeodomain-leucine zipper (HD-Zip) family proteins are unique to plants, but little is known about their role in defense responses. CaHB1 is a nuclear factor in peppers, belonging to subfamily II of HD-Zip proteins. Here, we determined the role of *CaHB1* in the defense response. *CaHB1* expression was induced when pepper plants were challenged with *Phytophthora capsici*, a plant pathogen to which peppers are susceptible, or environmental stresses such as drought and salt stimuli. *CaHB1* was also highly expressed in pepper leaves following application of SA, whereas ethephon and MeJA had a moderate effect. To further investigate the function of *CaHB1* in plants, we performed gain-of-function study by overexpression of *CaHB1* in tomato. *CaHB1*-transgenic tomatoes showed significant growth enhancement including increased leaf thickness and enlarged cell size (1.8-fold larger than control plants). Microscopic analysis revealed that leaves from *CaHB1*-transgenic plants had thicker cell walls and cuticle layers than those from controls. Moreover, *CaHB1*-transgenic plants displayed enhanced resistance against *Phytophthora infestans* and increased tolerance to salt stress. Additionally, RT-PCR analysis of *CaHB1*-transgenic tomatoes revealed constitutive up-regulation of multiple genes involved in plant defense and osmotic stress. Therefore, our findings suggest roles for *CaHB1* in development, salt stress, and pathogen defense.

1. Introduction

The homeodomain leucine zipper (HD-Zip) family of proteins in plants is a group of transcription factors that regulate development in response to environmental or external stresses [1,2]. The family is classified into four subfamilies (HD-Zip I-IV) by DNA-binding, conserved motif, and function [2]. The genes in subfamily I and II are involved in adaptation to external stimuli such as drought and salinity [3,4]. The H52 protein in tomato, a member of the HD-Zip family, limits cell death during pathogen infection [5]. Silencing of H52 in tomatoes resulted in cell death, which spread to uninfected neighboring plant parts during infection [5].

Osmotic treatment induced the expression of a number of HD-Zip family genes, namely *Arabidopsis thaliana HB6* (*AtHB6*), *Barssica napus HB6* (*BnHB6*), and *Oriza sativa hox22* (*Oshox22*) [6–8]. These genes were up-regulated by various environment stresses and by externally applied abscisic acid (ABA) or salicylic acid (SA) [6–8]. Zhang et al. [8] demonstrated that overexpression of *Oshox22* reduced tolerance to salt stress at the rice seedling stage through an ABA-mediated signaling pathway. However, there is no available information about the *Homeobox* (*HB*) gene-mediated defense response to pathogen attack.

Recently, we described the *NbHB1* gene, which encodes an HD-Zip protein from *Nicotiana benthamiana* [9]. Using transient or virus-induced gene silencing in *N. benthamiana*, we showed that *NbHB1* positively regulated pathogen-induced cell death. In our studies, expression of *NbHB1* following inoculation with pathogens and various other treatments was enhanced [9]. Ectopic expression of *NbHB1* accelerated cell death following abiotic stresses or bacterial pathogen inoculation [9]. According to protein databases, the NbHB1 protein belongs to subfamily II with an *N*-terminal variable region and *C*-terminal conserved CPSCE motif [1,9]. We also identified *Capsicum annuum homeobox 1* (*CaHB1*), an HD-Zip II family gene, in pepper plants infected with a bacterial pathogen [9]. However, the role of *CaHB1* in disease resistance is unknown.

Here, we describe the role of *CaHB1* in plant defense against pathogens using gain-of-function studies in transgenic tomato (*Solanum lycopersicon* L. cv. 'MicroTom') plants overexpressing

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CaHB1. We believe these results will considerably enhance understanding of the role of HD-Zip genes in defense against pathogens and environmental stresses.

2. Materials and methods

2.1. Plant materials

For tomato (*S. lycopersicon* L. cv. 'MicroTom') transformation, seeds were surface-sterilized in 1% (v/v) NaOCl, followed by wash with sterilized distilled water. Seeds were then germinated on MS agar medium and kept in a plant growth chamber under a 16-h photoperiod at 25 °C for 2 weeks before being used for transformation [10]. Tomato and pepper plants (cv. Bugang) were grown in pots and maintained under a 16-h photoperiod at 25 °C.

2.2. Agrobacterium-mediated transformation of tomato

A method for generation of transgenic plants was used as described by Oh et al. [10]. Briefly, cotyledons from 2-week-old tomato seedlings germinated on MS medium were used for co-cultivation with Agrobacterium to generate transgenic plants [10]. Kanamycin-selected transgenic plants were grown in a greenhouse, and then transgenic plants were further selected by measuring the expression of the nopaline synthase terminator by PCR with a primer set (Table S1).

2.3. Evaluation of CaHB1-transgenic tomato resistance to Phytophthora infestans

Four-week-old T3-tomato plants were inoculated with the zoospores of *P. infestans*. *P. infestans* infection of tomato plants were performed using zoospores inoculations as described by Oh et al. [10]. Disease symptoms appeared within 2 to 3-DAI from 5–6 independent infected plants. The index of *Phytophthora* infection was determined visually based on the necrotic leaf area [10].

2.4. Chlorophyll content analysis

For the leaf disk assay, tomato leaf disks with a diameter of 10 mm ($n = 5$) were prepared from tomato leaves of identical development stage of both vector-control and transgenic plants and floated on solutions of different NaCl concentrations for 3-days. After treatment with NaCl (0.5 and 1.0 M), chlorophyll was extracted from transgenic or control only transgenic-tomato leaves according to the method of Hu et al. [11], and then the chlorophyll content was calculated by the method of Lichtenthaler [12]. The photographs were taken at 3 days, and the experiments were repeated at least twice.

2.5. Microscopic analysis

Microscopic analysis was carried out following the methods as described by Sarowar et al. [13]. Leaves of one-month-old control or *CaHB1*-transgenic plants were observed under a light microscope and a transmission electron microscope (TEM).

2.6. Chemical treatment and pathogen inoculation

To determine the expression of the *CaHB1* transcripts after treatment of 5 mM SA, 5 mM ethephone, 100 μM MeJA, or 100 μM ABA, leaves of pepper plants were sprayed with chemicals as described in Oh et al. [10].

2.7. Subcellular localization of CaHB1 protein

A construct, CaHB1-smGFP under the control of CaMV-35S promoter, was made for CaHB1 expression in *N. benthamiana* protoplast. The fusion constructs were introduced into *N. benthamiana* protoplasts prepared from young leaves by the polyethylene glycol-mediated transformation Oh et al. [10]. Expression of the fusion constructs was observed at 40 h after transformation using a confocal laser scanning microscope (Carl Zeiss LSM 510), and the image was captured with a cooled charge-coupled device camera. The filter sets were used as described in Oh et al. [10].

2.8. RT-PCR analysis

Total RNA samples were extracted from pepper using TRI reagent according to the manufacturer's instructions (Invitrogen). RT-PCR was performed to detect the endogenous levels of several genes using the primer sets listed in Supplemental Table S1. The expression of the PiEF1a gene was controlled with a primer pair specific for the constitutively expressed tomato *Actin* gene (Table S1).

3. Results

3.1. Expression and cellular localization of CaHB1

We isolated *CaHB1* using genome-wide screening, which identifies genes up-regulated during pepper-bacterial pathogen interaction [9,14]. This gene's (NCBI accession No. EU998972) open reading frame encodes 272 amino acid residues (Fig. S1) and, according to protein databases, contains an N-terminal variable region, an HD-Zip domain, and a C-terminal conserved CPSCE motif. Therefore, it belongs to subfamily II of the HD-Zip family [1,9]. The overall structure of CaHB1 is similar to other HD-Zip family plant proteins, especially NbHB1, with which it shares 84% amino acid identity (Fig. S1).

The expression of *CaHB1* was determined in different tissues of the pepper plant. High levels of *CaHB1* transcripts were present in flowers and stems, while levels were low in leaves and roots (Fig. 1A). *CaHB1* was highly expressed during development in pepper leaves, indicting *CaHB1* regulates senescence (Fig. 1B).

HD-Zip proteins are transcription factors; therefore, we determined whether CaHB1 is present in the nucleus. Although we did not find putative nuclear localization sequences in CaHB1 with the PSORT II program (http://psort.nibb.ac.jp), other evidence indicates that it is localized in the nucleus. CaHB1 was tagged at the C-terminal with soluble modified green fluorescent protein (smGFP) and constitutively expressed using the CaMV-35S promoter (Fig. 1C). The fused construct was transformed into protoplasts of *N. benthamiana*, and GFP florescence was localized in the nucleus (Fig. 1C). In contrast, with the 35S-smGFP control, GFP fluorescence was observed throughout the cytoplasm and the nucleus. Like other HD-Zip proteins, CaHB1 appears to be a nuclear transcription factor.

3.2. Effect of biotic and abiotic stresses on CaHB1 expression

To understand the function of CaHB1 in stress, the expression levels of *CaHB1* were monitored in pepper plants treated with biotic and abiotic stress. *CaHB1* expression was strongly induced when pepper leaves were inoculated with the pepper pathogen, *Phytophthora capsici*. Likewise, *CaHB1* transcripts increased remarkably after inoculation, before disease symptoms were visible, and remained elevated until 72 h after inoculation (Fig. 1D). *CaPR-1* transcript, a marker gene, was detected 48 h after inoculation and remained high for 72 h (Fig. 1D).

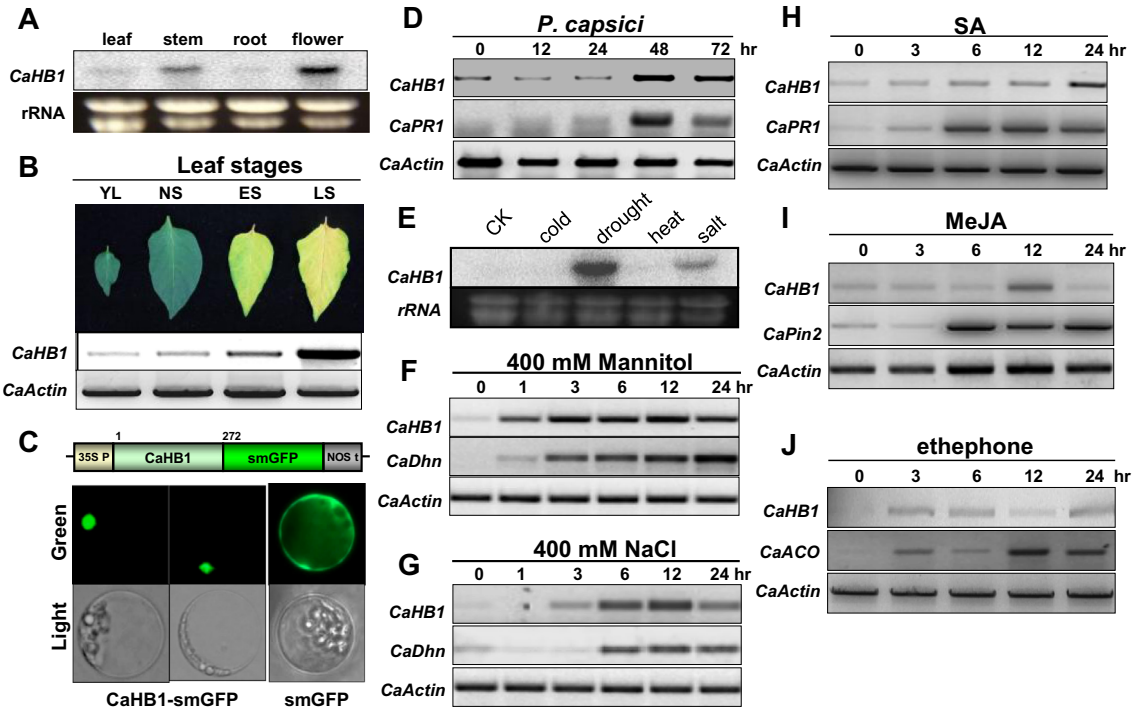

Fig. 1. Cellular localization and expression of *CaHB1* plant. (A) Expression of *CaHB1* in different tissues. RNAs from leaf, stem, root, and flower were used. (B) Expression of *CaHB1* transcripts in leaves during senescence. YL, young leaf; NS, fully expanded, non-senescent; ES, early senescent; LS, late senescent. (C) Localization of CaHB1 in the nucleus of *N. benthamiana* protoplasts. (D–J) Expression of *CaHB1* in response to pathogens and abiotic stresses. Pepper leaves were inoculated with *P. capsici* (D), and were treated with wounding, cold, drought, heat, salt (E), and treatment of 400 mM mannitol (F) or NaCl (G). (H–J) Expression of *CaHB1* mRNA in response to plant hormones including SA (H), MeJA (I), and ethephone (J), onto pepper leaves. *CaPR1*, *CaACO*, *CaPinII*, or *CaDhn* gene was used as a positive marker gene, respectively.

The expression of *CaHB1* was also assessed after cold, drought, heat, or salt stress. Expression of *CaHB1* was only detected in drought- or salt-treated leaves, implying that *CaHB1* regulates the response to these stresses (Fig. 1E). We next performed a time course of the expression of *CaHB1* after drought- or salt-stress. Exogenous mannitol was used to mimic drought. Three hours after mannitol treatment, *CaHB1* transcripts had accumulated markedly and the expression level was sustained until 24 h after treatment (Fig. 1F). Similar results were achieved in response to salt stress resulting from treatment with 400 mM NaCl; although, the maximum expression was detected 12 h after treatment (Fig. 1G). *CaDhn*, a marker gene for drought or salt stress, had an expression pattern similar to that of *CaHB1*.

Stress responses in plants are linked to hormonal regulation, so we assessed the effect of stress-related hormones on *CaHB1* expression. SA, ethylene, and JA are plant defense-related signal molecules [15,16] and ABA regulates responses to osmotic stress [7]. We treated pepper leaves with SA, ethephon (which will be converted to ethylene), methlyjasmonate (MeJA), or ABA and measured expression of *CaHB1*. SA (Fig. 1H), MeJA (Fig. 1I) or ethephon (Fig. 1J) induced the expression of *CaHB1*, while treatment with ABA had no effect (data not shown). These results may suggests that SA and ethylene may be signal molecules that modulate *CaHB1*-mediated responses.

3.3. Overexpression of CaHB1 in tomato resulted in a thicker cell wall

To evaluate the role of CaHB1 in plants, we used *Agrobacterium*-mediated transformation to generate transgenic-tomato plants that constitutively expressed *CaHB1* under the control of the CaMV-35S promoter. Eighteen *CaHB1*-transgenic lines showed strong expression and were selected. In the T3 generation, 11 *CaHB1*-transgenic tomato lines were larger than the empty-vector transformed plants at the same age. Enhanced growth in these transgenic lines was due to taller plants, slightly larger leaves, and longer internodes at the flowering stage. Three transgenic lines with stable expression of *CaHB1* were selected for further studies (Fig. 2A).

Microscopic analysis of cross sections of leaves of *CaHB1*-transgenic or control plants revealed that all *CaHB1*-transgenic tomato lines had significantly thicker leaves than control plants and the mesophyll cells of the transgenic lines were at least 1.3–1.6 times longer than those of control leaves (Fig. 2B). Transmission electron microscopy revealed that three of the *CaHB1*-overexpressing lines also had thicker cell walls and cuticles than control plants (Fig. 2C). The cell walls of the *CaHB1*-transgenic lines were 1.75–1.95 times thicker that those from control plants (Fig. 1D).

3.4. Overexpression of CaHB1 in tomato confers resistance to *P. infestans*

Next, we studied the role of the *CaHB1* gene in defense using the *CaHB1*-transgenic tomato plants infected with *P. infestans*. This pathogen causes tomato late blight disease and is the most devastating member of the oomycete genus *Phytophthora* [17]. Within 3-days after inoculation (DAI), disease symptoms were visible in both the control and *CaHB1*-transgenic tomato plants (Fig. 3A). However, the area of infection was much smaller in the transgenic lines than in the control plants (Fig. 3A). Three DAI visible disease symptoms were observed only in 11–16% of the total leaf area of the transgenic lines, compared to 41% of the total leaf area in the control plants (Fig. 3A).

EF2a gene expression in *P. infestans*-infected tomato leaves was measured to quantify the hyphae biomass present. At baseline and 4 DAI, quantitative RT-PCR analysis revealed that levels of *PiEF2a* transcripts were lower in the transgenic tomato lines than in the

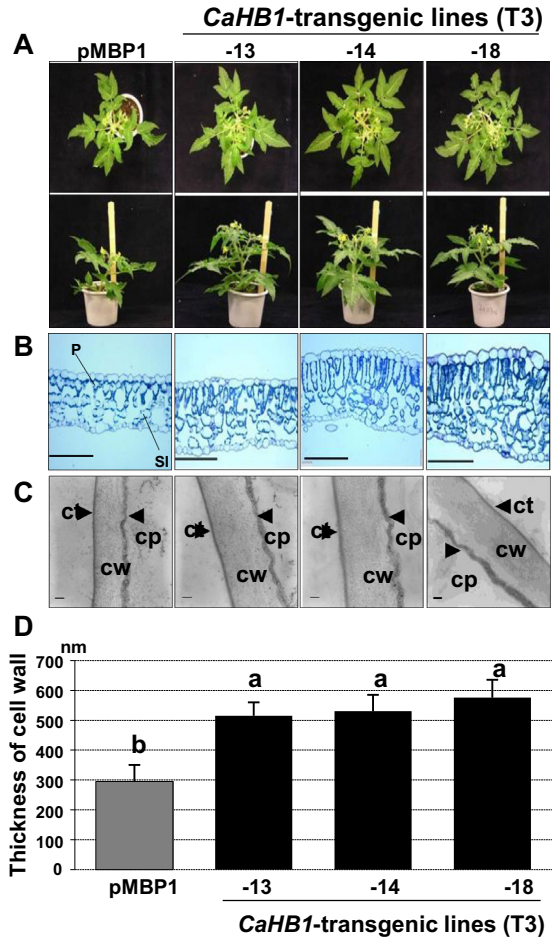

Fig. 2. Phenotypes of *CaHB1*-overexpressing tomato plants. (A) Growth enhancement by *CaHB1* overexpression in tomato plants. (B) Cytological and cellular analyses of the *CaHB1*-overexpressed plant. Light micrographs of leaf cross sections of control (pMBP1), *CaHB1-13*, *CaHB1-14*, and *CaHB1-18*. P, palisade cell layer, Sl, spongy mesophyll cell layer, Bar = 100 μm. (C) Transmission electron microscopy of the leaf epidermis cell wall of transgenic-tomato. Thirty-day-old leaves of control and three transgenic lines were cross-sectioned for imaging of epidermis cell walls. ct, cuticle; cw, cell wall; cp, cytoplasm. Bar = 0.1 μM. (D) Thickness of cell walls in the control and *CaHB1*-transgenic lines 13, 14, and 18. Values represent means and ±SD (letters; $P = 0.05$).

control plants. The tomato *Actin* gene (*SlActin*) was used to internal control of RNA quality (Fig. 3B). *PiEF2a* transcript levels in the transgenic plants were 2–4 times less than in the control plants (Fig. 3B). This may due to loss of viable tissue during the progress of the disease.

The effects of *CaHB1* overexpression in transgenic tomato on defense-related gene expression were determined using RT-PCR analyses for several tomato defense-related genes that are expressed during plant responses to pathogens and in response to SA [15,16]. Expression of *CaHB1* in tomatoes increased the abundance of the following transcripts: *SlPR1*, *SlGluA* (β-1,3-glucanase), *SlChi3* (chitinase), and *SlPR23* (Fig. 3B).

3.5. Overexpression of CaHB1 enhanced tolerance to saline stress in tomato

In an earlier study, the number of *CaHB1* transcripts increased during NaCl treatment, suggesting CaHB1 functions in the plant's response to salt stress. Thus, we next chose to explore further the role of CaHB1 in salt stress. The transgenic tomato plants and their control plants were used in the study as well as a leaf disc assay that has been demonstrated to be a reliable index of salt stress-induced damage to the photosynthesis apparatus [18]. Leaf discs from control and three *CaHB1*-transgenic lines were submerged in 0.5 M NaCl solution and exposed to light for 3-days. At this time, the control leaf discs were yellow, and the *CaHB1*-transgenic leaf disks remained green (Fig. 4A).

The chlorophyll content was measured in the discs after 3-days. There was no difference in the chlorophyll content of untreated discs from transgenic and control plants. In contrast, there was in the control leaf discs showed 28% chlorophyll loss, and *CaHB1*-transgenic discs had only 10% chlorophyll loss (Fig. 4B), suggesting that *CaHB1* is directly involved in the adaptational mechanism for tolerance to salt stress, functioning as an important transcription factor in this signaling pathway.

To investigate how *CaHB1* may have increased tolerance to salinity, the expression of some stress-related genes were analyzed. The transcripts levels of *Dehydrin* gene [19] in *CaHB1*-transgenic lines were up-regulated to levels 2–3 times that in control plants (Fig. 4C). Thus, the increased salt tolerance of *CaHB1*-overexpressing plants correlated with elevated expression levels of these genes, which have established roles in stress responses. This

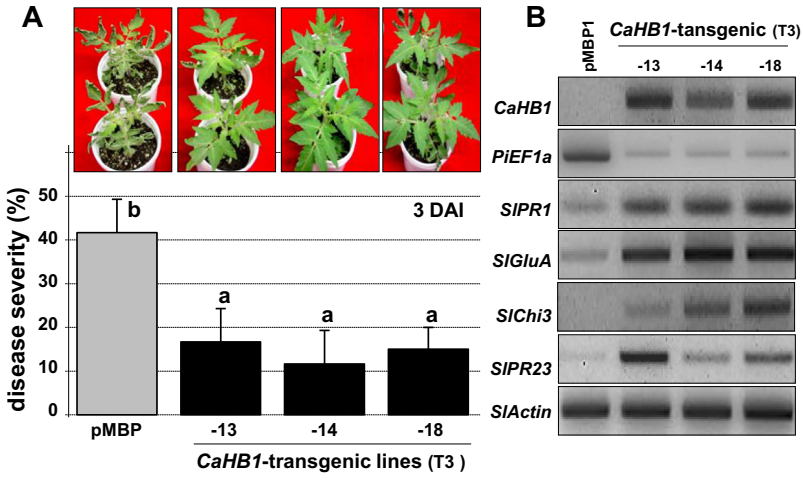

Fig. 3. Response of CaHB1-transgenic tomato plants against *P. infestans* infection. (A) Reduced infection frequency in *CaHB1*-transgenic tomato plants 3DAI after *P. infestans* infection. The control and *CaHB1*-transgenic lines -13, -14, and -18 were used (Upper panel). Disease symptoms of infection in the control and *CaHB1*-transgenic plants. Disease severity measured on 10-plants per line infection with *P. infestans* (Lower panel). Values represent means and ±SD (letters; $P = 0.05$). (B) RT-PCR analysis of defense-related genes (*SlPR1*, *SlGluA*, *SlChi3* and *SlPR23*) in *CaHB1*-transgenic plants. RT-PCR of *Phytophthora* colonization levels in *CaHB1*-expressing and control plants at 3DAI. *PiEF2a* expression was controlled with primer *SlActin* that is specific for the constitutively expressed tomato *Actin* gene.

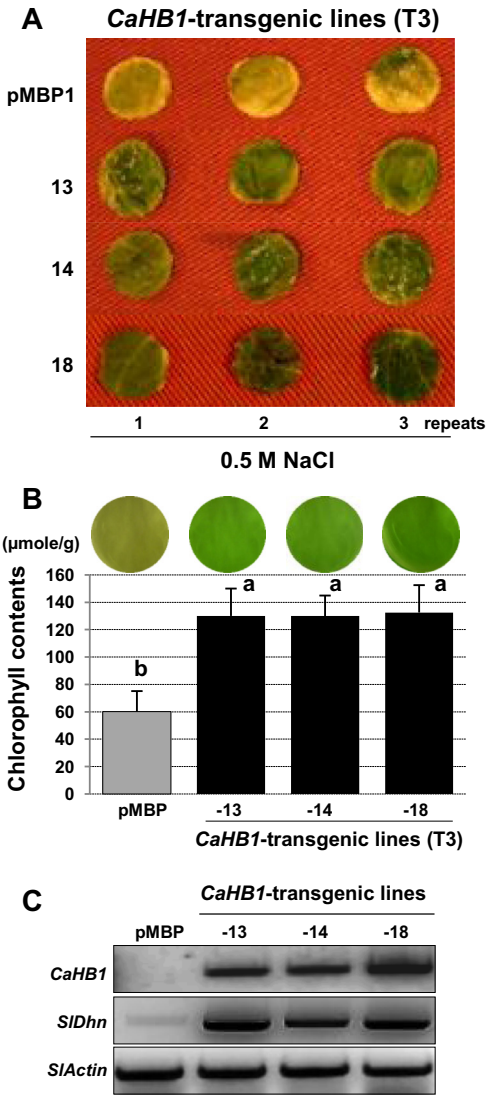

Fig. 4. Ectopic expression of CaHB1 and tolerance levels to salt stress. (A) Leaf discs of *CaHB1*-transgenic tomato (*CaHB1-13*, *CaHB1-14*, and *CaHB1-18*) following treatment with 0.5 M NaCl. Photographs were taken at 3-days after treatment. (B) Chlorophyll contents of *CaHB1*-ransgenic tomato plants following treatment with NaCl. Chlorophyll contents were measured 3-days after treatment with the indicated concentrations of NaCl, and photographs were taken at the same time. Values represent means and ±SD (letters; $P = 0.05$). (C) RT-PCR analysis of tomato *Dehydrin* gene in *CaHB1*-transgenic tomato plants. *SlActin* was used to confirm equal total RNA amounts among samples.

supports the suggestion that CaHB1 enhances salt tolerance in plants.

4. Discussion

In this study, we found that the HD-Zip factor II protein CaHB1 regulates plant responses to biotic and abiotic stress as well as development. We examined the roles of CaHB1 using *CaHB1*-overexpressing transgenic plants, and found that *CaHB1* regulates growth and development. Overexpression of *CaHB1* in tomato plants enhanced growth compared to control plants; transgenic plants were taller, had larger leaves with thicker cell walls, and had larger cells (Fig. 2D).

CaHB1 was expressed after exposure to *P. capsici* and up-regulated by exogenous SA and ET, which play intermediate roles in pathogen-induced signaling pathways. Recently, we reported that the HD-Zip gene *NbHB1* was induced by a compatible host response following infection with a bacterial pathogen; although, the role of *NbHB1* in plant resistance response was not elucidated [9]. Functional analysis of *CaHB1* using transgenic plants revealed that over-expression of the *CaHB1* in tomato increased host resistance against *P. infestans*. Although the role of *CaHB1* was completely not elucidated, changes in the physical barrier, notably the thickened cell wall and cuticle, which could prevent zoospore penetration, may have enhanced disease resistance to *P. infestans* inoculation. This result indicates that *CaHB1* plays an important role resistance to the oomycete pathogen. It was also found that the expression levels of several pathogenesis-related genes, including *SlPR1*, were higher in *CaHB1*-expressing plants than in the control plants. Thus, our findings provide strong evidence that CaHB1 plays a crucial role in the common defense response, including accumulation of pathogenesis related-genes, which is induced by general signaling pathways in plants.

Next, *CaHB1* expression was induced in response to abiotic stress. Pepper plants were treated with NaCl or mannitol, which mimics drought (Fig. 2D). Several HD-Zip proteins are induced by salt or drought stress [20–22], and these stress responses have been linked to ABA signaling [8,23]. However, *CaHB1* was not induced by ABA treatment in this study, implying that it may function independently of ABA signaling. There are also several reports that ET and SA are involved in drought stress [24,25]. ET or SA may induce *CaHB1* during salt or drought stress synergistically or independently. This hypothesis could be tested with mutants deficient in ET- or SA-signaling.

Moreover, we demonstrated that ectopic expression of *CaHB1* enhanced the tolerance of the transgenic tomato plants to salt stress. Our finding suggests that CaHB1 has promise in improving salinity tolerance in tomato plants. Chlorophyll is an essential factor for plant growth and development, and using a leaf disk assay to examine plants under salt stress, we found less chlorophyll loss in *CaHB1*-transgenic plants than in control plants. This indicates that *CaHB1*-transgenic tomatoes have higher photosynthetic capacity than that of control plants. Additionally, we also found that *Dehydrin* transcript level in *CaHB1*-transgenic lines were up-regulated 3- to 5-fold compared to levels in control plants. Therefore, it is possible that overexpression of *CaHB1* in tomato plants may enhance the salt tolerance.

Finally, considering the effect of ethylene or JA on *CaHB1* expression, it is possible that ethylene or JA modulates senescence at least partly through *CaHB1* activity. In studies of a close homolog of *CaHB1* in *N. benthamiana*, *NbHB1*, overexpression of *NbHB1* induced cell death following treatment of plants with MeJA or exposure to darkness [9] (Fig. S2). Positive regulation of senescence by JA signaling has been reported, including elevated JA levels during senescence, precocious senescence following treatment with JA, and the loss of JA-induced senescence by *coi1* mutation [26,27]. These studies suggest that CaHB1, along with ethylene or JA, regulates senescence.

CaHB1 was also highly induced by drought. However, it is possible that *CaHB1* accelerates drought-induced senescence. A recent report implies that drought and senescence are linked, and in this study, delayed senescence also rendered plants tolerant to drought [28]. As *CaHB1* was also induced during senescence, it may be involved in drought-induced senescence.

Our results suggest that overexpression of *CaHB1* in tomato plants activated various biological processes, including cell enlargement, cell-wall thickening, and induction of defense mechanisms. These biological processes may confer disease resistance against *Phytophthora*, tolerance to salt stress, and growth promotion. Thus, these studies suggest that expression of *CaHB1* is required for plant growth and development and for defense against oomycete pathogens.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.019.

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