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RESEARCH PAPER

Cell wall/vacuolar inhibitor of fructosidase 1 regulates ABA response and salt tolerance in Arabidopsis

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ABSTRACT

ABA regulates abiotic stress tolerance in plants via activating/repressing gene expression. However, the functions of many ABA response genes remained unknown. C/VIFs are proteinaceous inhibitors of the CWI and VI invertases. We report here the involvement of C/VIF1 in regulating ABA response and salt tolerance in Arabidopsis. We found that the expression level of *C/VIF1* was increased in response to ABA treatment. By using CRISPR/Cas9 gene editing, we generated transgene-free *c/vif1* mutants. We also generated *C/VIF1* overexpression plants by expressing *C/VIF1* under the control of the *35S* promoter. We examined ABA response of the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants by using seed germination and seedling greening assays, and found that the *35S:C/VIF1* transgenic plants showed an enhanced sensitivity to ABA treatment in both assays. On the other hand, the *c/vif1* mutants showed slight enhanced tolerance to ABA only at the early stage of germination. We also found that salt tolerance was reduced in the *35S:C/VIF1* transgenic plants in seed germination assays, but slightly increased in the *c/vif1* mutants. Taken together, our results suggest that *C/VIF1* is an ABA response gene, and C/VIF1 is involved in the regulation of ABA response and salt tolerance in Arabidopsis.

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Cell wall/vacuolar inhibitor of fructosidase 1; abscisic acid; salt tolerance; gene editing; Arabidopsis

Introduction

Abiotic stresses including drought, cold, salinity and extreme temperatures reduce the yield of crops and other plants.[1,2] ABA (abscisic acid) is the key plant hormone that regulates plant responses to abiotic stresses.[3-6] ABA signaling through the PYR1/PYL/RCAR (Pyrabactin resistance 1/PYR1-like/Regulatory component of ABA receptor) receptors,[7-9] the PP2Cs (A-group PROTEIN PHOSPHATASE 2Cs) phosphatases,[10,11] the SnRKs (NONFERMENTING 1 (SNF1)-RELATED PROTEIN KINASES) protein kinases,[12] and a few ABF/AREB/ABI5-type bZIP (basic region leucine zipper) transcription factors[13-15] regulate plant response to abiotic stresses via activating/repressing ABA response genes.[3,5,6,10-12] Consistent with this, mutation in the ABA singling genes affected plant tolerance to abiotic stresses. For example, overexpression of *PYL* genes enhanced drought tolerance in plants,[16,17] whereas loss-of-function of *SnRK* genes or ABF/AREB/ABI5-type bZIP transcription factor genes led to reduced drought tolerance in plant.[18,19] However, functions of a lot of ABA response genes in ABA signaling and/or plant abiotic stress responses remained unknown.

Cell wall invertase (CWI) and vacuolar invertase (VI) are acid invertases, they can irreversibly cleave sucrose, a primary form of carbon assimilates in the non-photosynthetic sink organs, into glucose and fructose.[20-23] The glucose and fructose then serve as metabolites and nutrient sources of the plants, and as signaling molecules to modulate gene expression during plant growth and development as well as plant responding to environmental cues.[24,25] As a result, up- or down-regulation of *CWI* and *VI* expression levels affected plant growth and development and/or plants response to stresses. For example, overexpression of *CWI* gene delayed leaf aging and enhanced drought tolerance in tobacco and tomato,[26,27] suppression of *VI* gene decreased cold-induced sweetening in potato,[28,29] and ectopic expression of a tea *VI* gene enhanced cold tolerance in Arabidopsis.[30]

Activities of CWI and VI invertases are regulated by the low-molecular-weight proteinaceous inhibitors, cell wall/vacuolar inhibitor of fructosidases (C/VIFs), who can physically interact with CWI and VI invertases and inhibit their activities.[31-33] Consistent with their biological functions, changes in the expression of *C/VIF* genes also affected plant growth and development and/or plants response to stresses. For example, silencing of *CIF* gene led to improved seed filling, prolonged leaf green, and enhanced cold tolerance in tomato,[34,35] loss-of-function of *CIF* gene resulted in increased seed production and germination in Arabidopsis,[36] and ectopic expression of tobacco *VIF* enhanced drought tolerance in Arabidopsis.[37] However, the relationship between ABA and C/VIF inhibitors in regulating plant abiotic tolerance remained largely unclear.

We report here the roles of C/VIF1 in regulating ABA response and salt tolerance in Arabidopsis. We found that the expression level of *C/VIF1* was upregulated by ABA, and transgenic plant overexpressing *C/VIF1* showed enhanced sensitivity to ABA and reduced tolerance to salt.

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Materials and methods

Plant materials and growth conditions

The Columbia-0 (Col) wild-type Arabidopsis (*Arabidopsis thaliana*) was used as a control for ABA sensitivity and salt tolerance assays, and for plant transformation to generate *c/vif1* mutants and *35S:C/VIF1* transgenic plants.

For plant transformation, seeds of the Col wild type were germinated and grown in soil pots in a growth room. For total RNA isolation, ABA response and salt tolerance assays, seeds of the Col wild type, the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants were sterilized with 25% (v/v) bleach for 10 min, washed with sterilized water four times, and placed on vitamins (PlantMedia)-containing 1/2 MS (Murashige & Skoog) plates. The medium contains 1% (w/v) sucrose and was solidified with 0.6% (w/v) phytoagar (PlantMedia). The plates were kept in darkness at 4°C for 2 d and then transferred to a growth room.

The growth conditions in the growth room were set at 22°C, and a 16 h light/8 h dark cycle with photon density at ~125 μmol m^{-2} s^{-1}.

ABA treatment, RNA isolation and RT-PCR

To examine the expression of *C/VIF1* and *C/VIF2* in response to ABA, 7-day-old Col wild-type seedlings were treated with 50 μM ABA or solvent methanol as a control for 4 h, frozen in liquid N_2. Total RNA was then isolated from the seedlings, and cDNA was synthesized as previously described.[38] RT-PCR was used to examine the expression of *C/VIF1* and *C/VIF2* in response to ABA treatment, and qRT-PCR was used to quantitative the fold changes of the expression levels. The expression of *ACT2* (*ACTIN2*) was used as a control. The primers used for PCR have been described previously.[36]

Constructs

To generate the *35 S:C/VIF1* construct for plant transformation, the full-length ORF sequence of *C/VIF1* was amplified by RT-PCR, double digested with *Nde1* and *Sac1* enzymes and cloned in frame with an N-terminal HA tag into the *pUC19* vector. The *pUC19-35S:C/VIF1* construct was then digested with *Pst1* and *Sac1* enzymes and cloned into the binary vector *pPZP211*,[39] to generate *pPZP-35S:C/VIF1* construct. The primers used to amplify *C/VIF1* were *C/VIF1-Nde1F*, 5′-CAACATATGATGAAGATGATGAAGGTGATGATG-3′, and *C/VIF1-Sac1R*, 5′- CGAGCTCTTACCAACAAGTTCTTCCTCTATTGAA-3′.

To generate *CRISPR/Cas9* constructs for *C/VIF1* gene editing, potential target sequences within the exons of *C/VIF1* were selected by using CRISPRscan (http://www.crisprscan.org/?page=sequence). Target specificity was then evaluated on Cas-OFFinder (http://www.rgenome.net/cas-offinder/). The specific target sequences selected for editing *C/VIF1* were 5′-GGTTCCTCTGCCGACACTTC(TGG)-3′, 5′-GTCCCCAAATTCGGCGAAGA(CGG)-3′ and 5′-GAAGACGGTGTGATCGACGC(CGG)-3′. We also selected one target for *C/VIF2*, i.e., 5′-AGTCCCACAGCCGACACAAA(AGG)-3′. The target sequences were inserted into the *pHEE-FT* vector,[40] by using a method as described by Wang et al.[41] The primers used to generate *pHEE-FT-C/VIF1* construct were *DT1-BsF* (*C/VIF1sgR1*), 5′-ATATATGGTCTCGATTGGTTCCTCTGCCGACACTTCGTT-3′, *DT1-F0* (*C/VIF1sgR1*), 5′-TGGTTCCTCTGCCGACACTTCGTTTTAGAGCTAGAAATAGC-3′, *DT2-R0* (*C/VIF1sgR2*), 5′-AACGCGTCGATCACACCGTCTTCAATCTCTTAGTCGACTCTAC-3′, and *DT2-BsR* (*C/VIF1sgR2*), 5′-ATTATTGGTCTCGAAACGCGTCGATCACACCGTCTTC-3′. The primers used to generate *pHEE-FT-C/VIF1-C/VIF2* construct were *DT1-BsF*(*C/VIF1sgR3*), 5′-ATATATGGTCTCGATTGTCCCCAAATTCGGCGAAGAGTT-3′, *DT1-F0* (*C/VIF1sgR3*), 5′-TGTCCCCAAATTCGGCGAAGAGTTTTAGAGCTAGAAATAGC-3′, *DT2-R0* (*C/VIF2sgR1*), 5′-AACTTTGTGTCGGCTGTGGGACCAATCTCTTAGTCGACTCTAC-3′, and *DT2-BsR* (*C/VIF2sgR1*), 5′-ATTATTGGTCTCGAAACTTTGTGTCGGCTGTGGGACC-3′. The *U626-IDF* and *U629-IDR* primers[42] were used for colony PCR and sequencing of the *CRISPR/Cas9* constructs generated.

Plant transformation, transgenic plant selection and transgene-free mutant isolation

The Col wild-type plants with several mature flowers (~5-week-old under our growth conditions) were transformed via *GV3101* mediated transformation by using floral dip method.[43] The plants were transformed with the *pPZP-35S:C/VIF1* construct to generate *35S:C/VIF1* overexpression plants, and the *CRISPR/Cas9* constructs to generate *c/vif1* mutants.

The *35S:C/VIF1* transgenic plants were selected by germinating the T1 seeds on 1/2 MS plates containing 50 μg/ml Kanamycin and 100 μg/ml Carbenicillin. The *35S:C/VIF1* transgenic plants with a single T-DNA insertion were selected by germinating T2 seeds, whereas homozygous *35S:C/VIF1* transgenic plants were selected by germinating T3 seeds on1/2 MS plates containing 30 μg/ml Kanamycin. Multiple homozygous lines were obtained and two with high expression levels of *C/VIF1* were used for the experiments.

Transgenic plants generated with the *CRISPR/Cas9* constructs were selected by germinating the T1 seeds on 1/2 MS plates containing 30 μg/ml Hygromycin and 100 μg/ml Carbenicillin, and transgene-free mutants were isolated as described previously.[40] In brief, early flowering T1 plants were selected and gene editing status was examined by amplifying and sequencing the genomic sequence of *C/VIF1*. T2 plants with normal flowering time were selected, and transgene-free status was confirmed by PCR amplification of *Cas9* gene fragment.

DNA isolation and PCR

To examine *C/VIF1* editing status, DNA was isolated from leaves of early flowering T1 or normal flowering T2 transgenic plants generated using the *CRISPR/Cas9* constructs, genomic sequences of *C/VIF1* were amplified by PCR and sequenced. To isolate transgene-free mutants, DNA was isolated from leaves of normal flowering T2 transgenic plants and used for PCR amplification of the *Cas9* gene fragment. The primers for amplifying *Cas9* fragment have been described previously.[42]

In planta invertase activity analysis

Invertase activity was analyzed by NBT (nitroblue tetrazolium) staining as described previously.[36] Briefly, seeds of the Col wild type and the *c/vif1* mutants were fixed with fixation buffer, rinsed with water to remove soluble sugar, and stained with staining solution. After staining, the seeds were rinsed with water and photographed under a dissection microscopy equipped with a digital camera.

ABA and salt sensitivity assays

ABA inhibited seed germination and cotyledon greening assays were performed as described previously.[44] Briefly, seeds of the Col wild type, the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants were sterilized and germinated on 1/2 MS plates in the presence or absence of 0.75 μM ABA. The plates were kept in darkness at 4°C for 2 d and then transferred to a growth room. Seeds germinated were counted every 12 h, photographs were taken and seedlings with green cotyledons were counted 8 d after the transfer. Percentage of germination and green seedlings was calculated.

For salt tolerance assays, seeds of the Col wild type, the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants were sterilized and germinated on 1/2 MS plates in the presence or absence of 200 mM NaCl. The plates were transferred to a growth room after kept for 2 d in darkness at 4°C. Seeds germinated were counted every 12 h, and the percentage of germination was calculated. All the experiments were repeated at least three times.

Results

Expression of *C/VIF1* is upregulated by ABA treatment

ABA plays an important role in regulating plant response to abiotic stresses. In order to identify novel players in ABA signaling and/or abiotic stress tolerance, we attempted to characterize the ABA response genes identified in our previously transcriptome assays, and with unknown functions or functions in ABA signaling and/or abiotic stresses remained unknown.[45] By using this strategy, we have previously successfully identified bHLH129 transcription factor as an ABA response regulator,[6,46] and AITRs as a novel family of transcription repressors that play a feed-forward regulating role in ABA signaling and function as negative regulators of plant response to abiotic stresses such as drought and salt.[6]

In the previous transcriptome data set, we found that the expression level of the invertase inhibitor gene *C/VIF1* was greatly increased in response to ABA treatment, with an RPKM of 22 in ABA treated seedlings compared with 2 in control samples. In another report, it has also been shown that the expression level of *C/VIF1* was slightly increased in response to ABA treatment.[36]

Therefore, we examined the expression of *C/VIF1* in response to ABA in Arabidopsis seedlings. The Col wild-type seedlings were treated with 50 μM ABA and RT-PCR was used to examine the expression of *C/VIF1*. We found that the expression level of *C/VIF1* was clearly increased in Arabidopsis seedlings treated with ABA (Figure 1a). Quantitative RT-PCR results showed that the expression level of *C/VIF1* in Arabidopsis seedlings increased about 10 folds in response to ABA (Figure 1b). On the other hand, the expression level of another known invertase inhibitor gene, *C/VIF2* remained largely unchanged (Figure 1).

Functions of C/VIF1 is affected in the gene editedc/vif1 mutants

Having shown that expression of *C/VIF1* was greatly upregulated by ABA treatment (Figure 1), we wanted to explore the functions of C/VIF1 in ABA response and abiotic tolerance in Arabidopsis by generating and analyzing overexpression plants and loss-of-function mutants of the *C/VIF1*. Transgenic plants overexpressing *C/VIF1* were generated by expressing *C/VIF1* under the control of the *35S* promoter (*35S:C/VIF1*) in the Col wild-type plants, and *CRISPR/Cas9* gene editing was used to generate *c/vif1* mutants.

Since we have used the *FT* expression cassette-containing *CRISPR/Cas9* construct *pHEE-FT* to generated transgene-free Arabidopsis mutants in a much fast and easier way,[40] the *pHEE-FT* vector was used to generate *CRISPR/Cas9* constructs for *C/VIF1* gene editing. Two *pHEE-FT* vector constructs were generated. The *pHEE-FT-C/VIF1* construct was

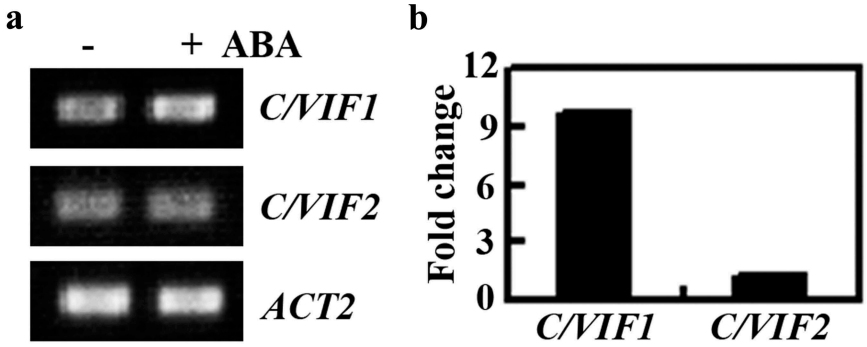

Figure 1. Expression of *C/VIF1* in response to ABA treatment. (a) Expression of *C/VIF1* was induced by ABA. Seven-day-old Col seedlings were treated with 50 μM ABA or mock-treated with solvent methanol for 4 h. Total RNA was isolated, cDNA was synthesized and used as a template for RT-PCR. The expression of *ACT2* was used as a control. (b) Fold changes of *C/VIF1* in response to ABA. Expression level of *C/VIF1* was examined by qRT-PCR. The expression of *ACT2* was used as an inner control. The fold change was calculated by comparing the expression levels of *C/VIF1* in ABA treated and mock-treated seedlings. Data represent the mean ± SD of three replicates.

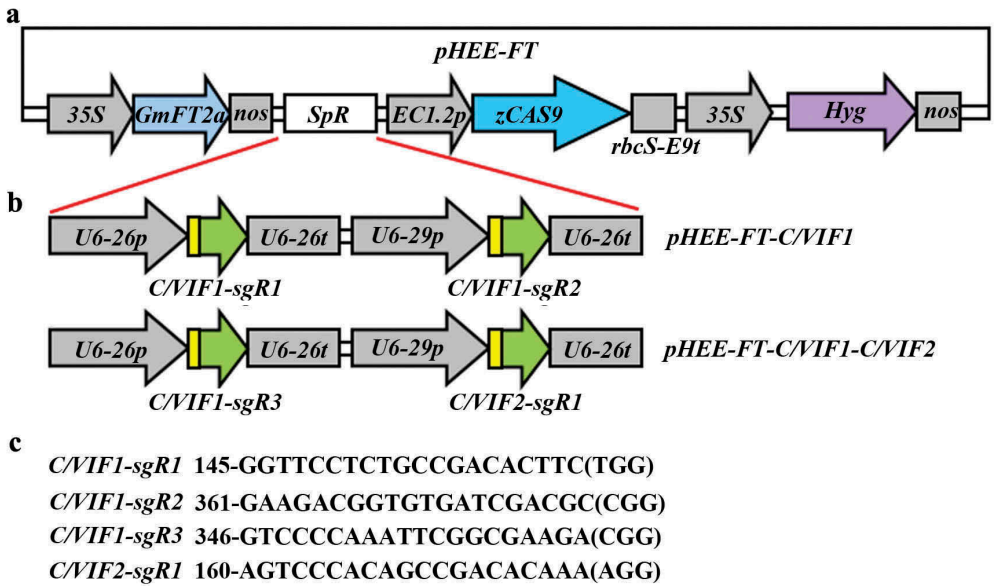

Figure 2. Generation of *pHEE-FT* CRISPR/Cas9 constructs for *C/VIF1* gene editing. (a) Diagram of the *pHEE-FT* vector with a *GmFT2a* expression cassette. (b) The *sgRNA* expression cassettes in the *pHEE-FT-C/VIF1* and *pHEE-FT-C/VIF1-C/VIF2* constructs. The target sequences of *C/VIF1* and *C/VIF2* were introduced into the *sgRNA* expression cassettes by PCR amplification, and the *sgRNA* expression cassettes were cloned into the *pHEE-FT* vector by using Golden gate cloning. (c) Target sequences in *C/VIF1* and *C/VIF2*. Numbers indicate the positions of the nucleotides in the coding sequences of *C/VIF1* or *C/VIF2*. PAM sites were indicated in the brackets.

made to target *C/VIF1* gene at two different sites, aimed to generate fragment deletion mutants. The *pHEE-FT-C/VIF1-C/VIF2* construct was made to target one target in *C/VIF1*gene, and the other in *C/VIF2*, intended to generate double mutants (Figure 2).

We obtained *c/vif1* single mutants with both constructs. The mutant generated by using *pHEE-FT-C/VIF1-C/VIF2* construct, *c/vif1-c1*, has a single nucleotide insertion occurred in *C/VIF1* (Figure 3a), led to a few amino acid substitutions and premature stop in C/VIF1 (Figure 3b). The premature stop occurred after PKF (Figure 3b), a motif has been shown to be critical for invertase–inhibitor interaction.[32] We also obtained another *c/vif1* mutant by using the *pHEE-FT-C/VIF1* construct, *c/vif1-c2*, which has 216 nucleotides fragment deletion in the coding sequence of *C/VIF1* (Figure 3a), resulted in 73 amino acids deletion spanning the PKF domain in C/VIF1 (Figure 3b). NBT (nitroblue tetrazolium) staining of the seeds showed that the invertase activities were increased in both *c/vif1-c1* and *c/vif1-c2* mutants (Figure 3c), suggest that the function of C/VIF1 was affected in these *c/vif1* mutants.

The *35S:CVIF1* transgenic plants are hypersensitive to ABA

After generated the *C/VIF1* overexpression and the *c/vif1* mutant plants, we examined their ABA response by using seed germination and cotyledon greening assays.

As shown in Figure 4a, reduced germination rate was observed for the *35S:C/VIF1* transgenic plant seeds at nearly all the time points examined, indicating that the *35S:C/VIF1* transgenic plants are more sensitive to ABA. On the other hand, a little difference on germination rate between the seeds of Col wild type and the *c/vif1-c2* mutant plants was observed at the beginning, however, reduced germination rate was observed for the *c/vif1* mutant plant seeds at the other time points examined (Figure 4a). The results suggest that disruption of the functions of C/VIF1 affected plant response to ABA.

In the cotyledon greening assays, the *35S:C/VIF1* plants also showed enhanced sensitivity to ABA treatment, but a little, if any difference was observed for the *c/vif1* mutants when compared with the Col wild-type plants (Figure 4b). Quantitative analysis showed that the green cotyledon rate of the *35S:C/VIF1* plants was only about half of the Col wild-type plants, whereas a slight decreased green cotyledon rate was observed for the *c/vif1* mutants (Figure 4c).

Salt tolerance is decreased in the *35S:C/VIF1* transgenic plants

ABA is a key hormone that affects plant response to abiotic stresses, having shown that C/VIF1 regulates ABA response in Arabidopsis, we further examined if C/VIF1 may also regulate plant response to abiotic stresses. We found that reduced germination rate was observed for the *35S:C/VIF1* transgenic plant seeds at nearly all the time points examined, suggest that salt tolerance is reduced in the *35S:C/VIF1* transgenic plants in the seed germination assays, whereas germination rate was slightly increased in the *c/vif1-c2* mutant seeds, but the *c/vif1-c1* mutants showed a near wild-type response to salt treatments (Figure 5).

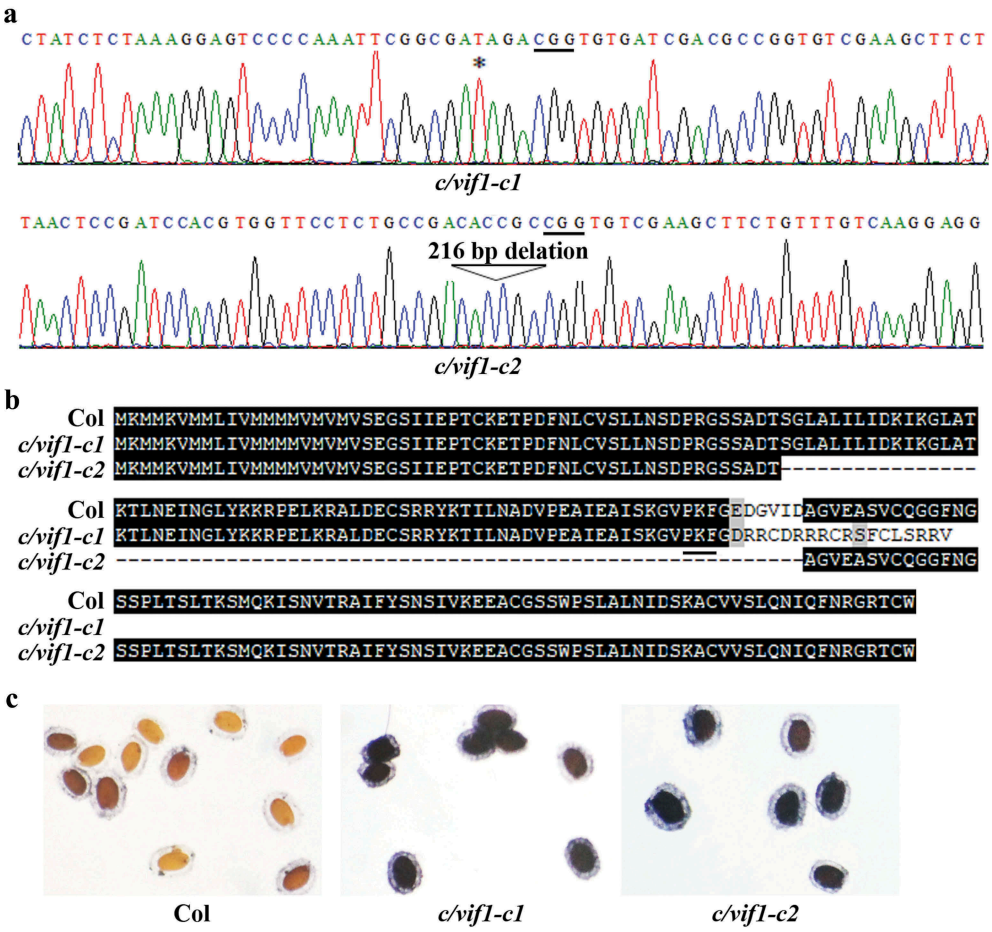

Figure 3. Isolation of transgene-free *c/vif1* mutants. (a) Editing status of *C/VIF1* in the *c/vif1* mutants. The *c/vif1-c1* and *c/vif1-c2* mutants were obtained by transforming Col wild-type plants with *pHEE-FT-C/VIF1-C/VIF2* and *pHEE-FT-C/VIF1* constructs, respectively. Editing status in T1 generation was examined, and transgene-free homozygous mutants were isolated in T2 generation. Star indicates the single T nucleotide insertion in the *c/vif1-c1* mutant. Arrowhead indicates the 216 bp fragment deletion in the *c/vif1-c2* mutant. Underlines indicate the PAM sites. (b) Alignment of C/VIF1 amino acid sequences in the Col wild type and the *c/vif1* mutants. ORF of *C/VIF1* in the *c/vif1* mutants was identified by using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) and predicted amino acid sequences were used for alignment with the amino acid sequence of C/VIF1. Underline indicates the PKF motif. (c) NBT staining of the seeds. Seeds of the Col wild type and the *c/vif1* mutants were fixed with fixation buffer for 1 h at 4°C, rinsed overnight with water to remove soluble sugar, and stained with staining buffer in a water bath at 30°C. Stained seeds were rinsed with water and photographed under a dissection microscopy equipped with a digital camera.

Discussion

C/VIFs are low-molecular-weight proteinaceous inhibitors that can inhibit the activities of CWI and VI,[31] both are acid invertases that can irreversibly cleave sucrose, into glucose and fructose.[20-23] Consistent with their roles in sucrose metabolism, accumulated evidence suggests that changes in *C/VIF* gene expression affect plant growth and development, as well as plants response to stresses including biotic stresses and abiotic stresses in several different plants such as potato,[47-50] tomato,[34,35,51] poplar,[33] and Arabidopsis.[36,37] Evidences provided in this study suggest that C/VIF1 plays a role in the regulation of ABA response and salt tolerance in Arabidopsis. First, the expression of *C/VIF1* was increased in response to ABA treatment (Figure 1). Second, the *35S:C/VIF1* transgenic plants showed enhanced sensitivity to ABA in both seed germination and seedling greening assays (Figure 4). Third, the *35S:C/VIF1* transgenic plants showed a reduced salt tolerance in seed germination assay (Figure 5).

In addition to serve as metabolites and nutrient sources of the plants, glucose and fructose also serve as signaling molecules to modulate gene expression during plant growth and development, and plant response to environmental stimuli,[24,25] whereas CWI and VI are able to cleave sucrose into glucose and fructose.[20-23] Therefore it is reasonable to speculate that C/VIF1's functions in regulating salt response is, at least partially, due to its ability in inhibiting CWI and VI activities, therefore affected the cleaving of sucrose into glucose and fructose. On the other hand, considering that ABA sensitivity in the *35S:C/VIF1* transgenic plants was affected (Figure 4), it will be of great interest to examine if and how ABA signaling may also play a role in C/VIF1 mediated salt tolerance in Arabidopsis. Considering that salinity affects ion content in plants, it will be also of

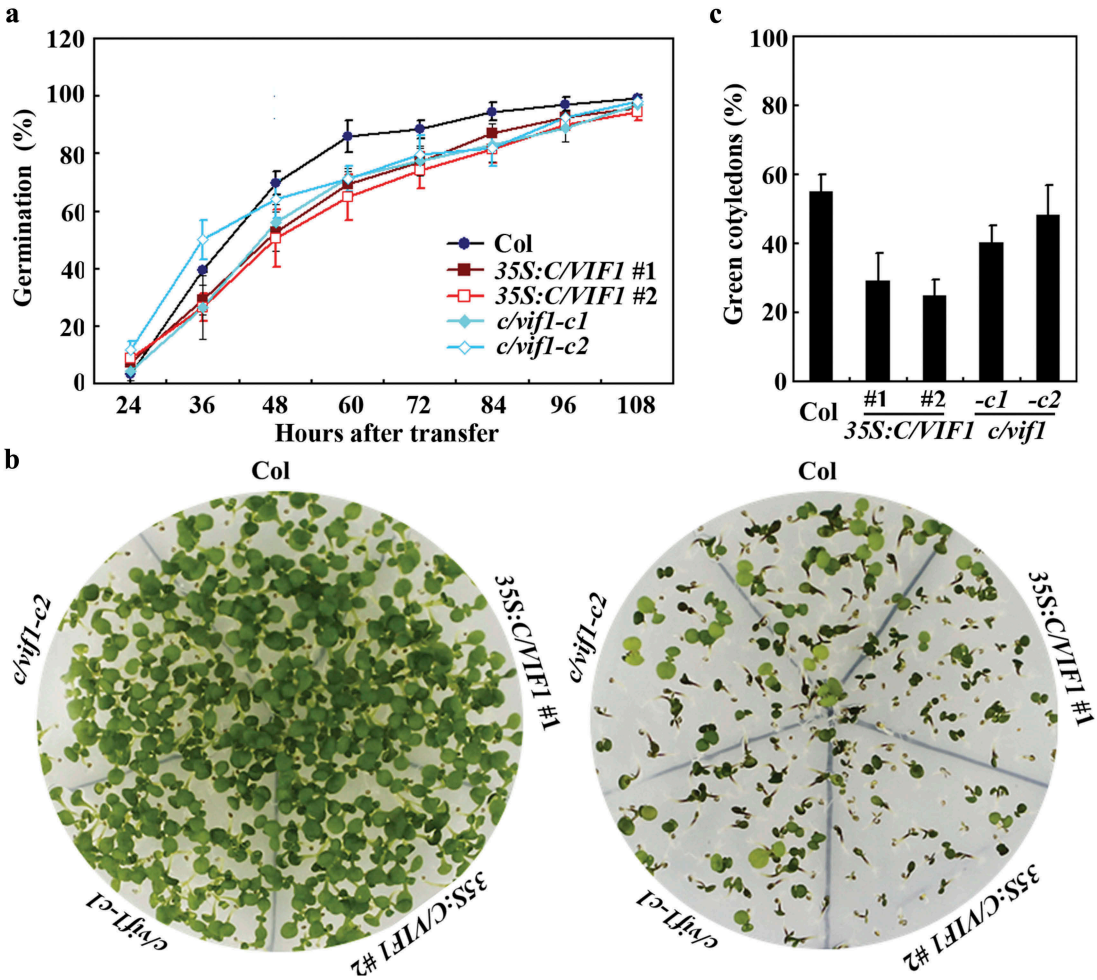

Figure 4. Effects of ABA on seed germination and cotyledon greening of the Col wild type, the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants. (a) Effects of ABA on seed germination of the Col wild type, the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants. Sterilized seeds of the Col wild type, the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants were germinated on 1/2 MS plates in the presence or absence of 0.75 μM ABA. The plates were transferred to a growth room after kept in darkness for 2 d at 4°C. Seeds germinated were counted every 12 h and the percentage of germination was then calculated. All the seeds on the control plates were generated 24 h after the transfer. Data represent the mean ± SD of three replicates. (b) Effects of ABA on cotyledon greening of the Col wild type, the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants. Sterilized seeds of the Col wild type, the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants were germinated on 1/2 MS plates in the presence or absence of 0.75 μM ABA. The plates were transferred to a growth room after kept in darkness for 2 d at 4°C, and pictures were taken 8 d after the transfer. (c) Percentage of green seedlings of the Col wild type, the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants in response to ABA treatment. Seedlings with green cotyledons were counted 8 d after the transfer and used to calculate the percentage of green cotyledons. Data represent the mean ± SD of three replicates.

interest to examine if there is any difference in ion contents in the Col wild type, the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants in response to salt treatment.

We noted that slightly reduced sensitivity to ABA was observed only in the *c/vif1-c2*, but not the *c/vif1-c1* mutants (Figure 4), similar, slightly enhanced salt tolerance was also observed only in the *c/vif1-c2* mutants (Figure 5). It has been reported that CIF interacts with CWI via its PKF motif, suggesting that C/VIFs inhibit invertase activities via direct physical interactions.[32,52] Considering that the single nucleotide insertion in the *c/vif1-c1* mutant resulted in a few amino acid substitutions and premature stop for C/VIF1 after the PKF motif, whereas the 216 nucleotides fragment deletion in the *c/vif1-c2* mutant resulted in 73 amino acids deletion of the PKF domain in C/VIF1 (Figure 3), it is very likely that the *c/vif1-c2* is a loss-of-function mutant, but the *c/vif1-c1* is not. However, because the invertase activities were also increased in the *c/vif1-c1* mutant (Figure 3), it is possible that C/VIF1 in the *c/vif1-c1* mutant may still able to interact with invertases, but its activities in inhibiting invertases were reduced. Even though additional evidence may be required to support that tolerance to ABA and salt was increased in loss-of-function mutants of *C/VIF1*, our evidence with the *35S:C/VIF1* transgenic plants indeed indicate that C/VIF1 plays a negative role in ABA response and salt tolerance.

It should be noted that C/VIF may have opposite effects in regulating abiotic stress responses in different plants. For example, silencing of *CIF* enhanced cold tolerance in tomato,[35] but ectopic expression of *VIF* enhanced drought tolerance in Arabidopsis.[37] However, silencing of *C/VIF* can improve agronomic traits in all plants studied so far. For example, silencing of *CIF* improved seed filling in tomato,[34] loss-of-function of *CIF1* increased seed production in Arabidopsis,[36] and suppression of *C/VIF* genes by *RNAi* improved seed weight in soybean.[53] Considering that CRISPR/Cas9 gene editing is able to generate transgene-free

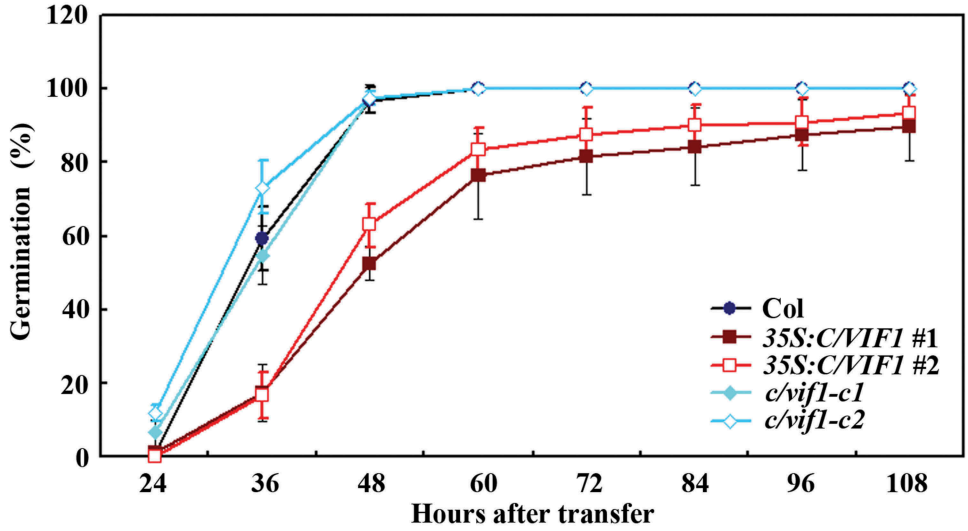

Figure 5. Effects of NaCl on seed germination of the Col wild type, the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants. Sterilized seeds of the Col wild type, the *35S:C/VIF1* transgenic plants and the *c/vif1* mutants were germinated on 1/2 MS plates in the presence or absence of 200 mM NaCl. The plates were transferred to a growth room after kept in darkness for 2 d at 4°C. Seeds germinated were counted every 12 h and the percentage of germination was then calculated. All the seeds on the control plates generated 24 h after the transfer. Data represent the mean ± SD of three replicates.

mutants and has been used to improve important agronomic traits in crops,[54-56] editing of *C/VIF* genes together with other agronomic trait controlling genes by CRISPR/Cas9 may enable to integrate desired agronomic traits in plants.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

SW conceived the study. SW, WY designed the experiments. WY, SC, YC, NZ, YM, WW, HT, YL and SH performed the experiments. WY and SW analyzed the data. SW drafted the manuscript. All the authors participated in the revision of the manuscript.

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