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ORIGINAL ARTICLE

Ectopic expression of *AtICE1* and *OsICE1* transcription factor delays stress-induced senescence and improves tolerance to abiotic stresses in tobacco

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Abstract Inducer of CBF Expression 1 (ICE1) is an upstream regulator of cold-responsive genes in *Arabidopsis thaliana* and various crop plants. The *ice1* mutant is impaired in expression of several transcription factors (TFs) and other genes involved in abiotic stress signaling, suggesting a role beyond cold tolerance. In this study, we examined the abiotic stress responses of transgenic *Arabidopsis thaliana* constitutively over-expressing *AtICE1*. These transgenic plants exhibited enhanced tolerance to NaCl and methyl viologen (MV)-induced oxidative stresses. To confirm this enhanced tolerance of *ICE1* in a heterologous system, transgenic tobacco over-expressing *AtICE1* and *OsICE1* were developed. *ICE1* over-expressing tobacco transgenic exhibited tolerance to NaCl- and PEG-induced stresses at seedling stage. The tobacco transgenic plants exhibited tolerant phenotype under the situations that mimicked natural drought condition. The improved drought tolerance observed in this study is linked to the maintenance of cell membrane stability and inhibition of stress-induced senescence. Higher expression of homologs of *ICE1* target genes such as *COR47*, *HVA22*, *ERD10*, *HSF1*, *EREBP3* and reduced expression of *WRKY6*, a positive regulator of senescence, indicated that *ICE1* expression activated diverse cellular tolerance mechanisms. The study demonstrated that *ICE1* is an important positive regulator of cellular tolerance mechanisms associated with multiple abiotic stress response.

Keywords Abiotic stress · Oxidative stress · Drought tolerance · Senescence · Transcription factor · ICE1

Abbreviations

ICE1	Inducer of CBF Expression 1
CBF	C-repeat/dehydration-responsive element Binding Factor
bHLH	Basic Helix-Loop-Helix
COR	COld-Regulated genes
ERD	Early Responsive to Dehydration
HSF	Heat Shock Factor
EREBP	Ethylene Responsive Element Binding Protein

Introduction

Abiotic stresses cause significant loss in global agricultural productivity and the yield losses in major crops are up to an extent of 60 % (Boyer 1982). Plants employ diverse stress adaptive mechanisms to survive and reproduce under stress conditions. Some of the whole plant and cellular level response mechanisms to stresses have been unraveled. During the past one decade significant progress has been made in unraveling abiotic stress signaling pathways, identification of signaling components and transcription regulators (Chinnusamy et al. 2010). Most signaling events lead to expression and/or activation of diverse transcription factors (TFs) belonging to the families of dehydration responsive element (DRE) binding factor/c-repeat (CRT) binding

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factor (CBF), basic Leucine Zipper (bZIP), basic helix-loop-helix (bHLH), calmodulin-binding transcriptional activator (CAMTA), no apical meristem (NAM), ATAF1-2 and cup shaped cotyledon (CUC) (NAC), nuclear factor-Y (NF-Y), WRKY, myelocytomatosis (MYC), myeloblastosis (MYB), and zinc finger homeo domain (ZFHD), which in turn orchestrate the expression of effector genes involved in stress acclimation (Yang et al. 2010). Over-expression of some of these TFs in isolation or in combination improved stress tolerance in different plants (Karaba et al. 2007; Lata and Prasad 2011; Pruthvi et al. 2014).

Amongst wide set of stress responsive TFs, CBFs are considered as an important class of proteins involved in cold acclimation in *Arabidopsis*. Low temperature induces the expression of CBF3/DREB1, which in turn activates several genes involved in cold acclimation (Liu et al. 1998). An important TF, designated as Inducer of CBF Expression 1 (ICE1) is a bHLH protein that is constitutively expressed and localized in the nucleus (Chinnusamy et al. 2003). ICE1 is a positive regulator of CBF genes. In *Arabidopsis*, cold stress activates ICE1, and the activated ICE1 binds to the promoter of CBF3 to induce its expression (Chinnusamy et al. 2003, 2010). Under cold stress ICE1 protein stability is negatively regulated by high expression of osmotically responsive gene 1 (*HOS1*), a RING finger ubiquitin E3 ligases (Dong et al. 2006) and positively regulated by sumoylation mediated by a SUMO E3 ligase, SIZ1 (SAP and Miz1) in *Arabidopsis* (Miura et al. 2007). Over-expression of wild-type ICE1 protein (Chinnusamy et al. 2003) or its mutant version with high trans-activation stability and low proteolysis enhanced the freezing tolerance in *Arabidopsis* (Miura et al. 2007). ICE1 also negatively regulates *MYB15*, a MYB TF that binds to CBF3 promoter and represses the expression (Agarwal et al. 2006). Several studies indicated that the CBF cold response pathway is conserved across plant species and over-expression of CBF3/DREB1 improves tolerance to multiple abiotic stresses in plants (Chinnusamy et al. 2010; Lata and Prasad 2011).

Constitutive over-expression of *AtICE1* resulted in enhanced osmolyte accumulation and cold tolerance in cucumber and rice (Liu et al. 2010; Xiang et al. 2008). Abscisic acid (ABA) and salt stress up-regulated the *ICE1* expression (Chinnusamy et al. 2003), and *ice1* mutant showed ABA-induced expression of $P_{CBF3::LUC}$ reporter (Chinnusamy et al. 2003). Additionally, constitutive or stress inducible expression of the *ICE1* target, CBF3, has been shown to impart multiple abiotic stress tolerance in several plant species (Lata and Prasad 2011). The expression of CBFs is induced by ABA, although to a lower level as compared with cold induction (Knight et al. 2004). These studies suggested that abiotic stress induced expression of CBFs may be at least in part under the control of *ICE1* regulation (Chinnusamy et al. 2006) and *ICE1* over-expression may improve tolerance to other abiotic stresses apart from cold-stress. *Oryza rufipogon bHLH2* gene, a homolog of *AtICE1*, conferred enhanced salt tolerance when constitutively expressed in *Arabidopsis* (Zhou et al. 2009). In this study we are demonstrating the role of *ICE1* in imparting salinity, desiccation and drought stress tolerance using transgenic tobacco constitutively over-expressing *OsICE1* and *AtICE1*. We show that ICE1 has roles in minimizing oxidative stress effects and also delaying leaf senescence.

Materials and methods

Analysis of abiotic stress tolerance of *Arabidopsis* plants over-expressing *AtICE1*

Arabidopsis thaliana transgenic seeds over-expressing *AtICE1* gene under the control of strong constitutive super promoter generated earlier (Chinnusamy et al. 2003) were used to examine the role of *ICE1* in different abiotic stress tolerance. Seeds were surface sterilized and inoculated on basal Murashige and Skoog (MS; Murashige and Skoog 1962) media and stratified at 4° C for two days. To assess the stress tolerance, five days old seedlings of wild-type (WT) and *AtICE1* over-expressing transgenic plants were transplanted onto MS media supplemented with NaCl (100 mM) and growth was analyzed after seven days. Total plant biomass was recorded after the stress treatment. Simultaneously, to evaluate oxidative stress tolerance, five days old seedlings of WT and *AtICE1* over-expressers were transplanted onto MS media supplemented with methyl viologen (MV; 5 μM). Stress tolerance was assessed by visible phenotype seven days after the imposition of stress.

Construction of *AtICE1* and *OsICE1* expression vectors and transformation of tobacco

Full-length cDNA clones of *OsICE1* (AK102594; Os01g0928000) and *AtICE1* (At3g26744) were obtained from Rice Genomic Research Centre (RGRC, www.rgrc.dna.affrc.go.jp) and (RIKEN, www.bgrc.riken.jp) Japan, respectively. Both the inserts were amplified using proof reading Phusion™ DNA polymerase (Finnzymes, USA). Gateway cloning® strategy (Invitrogen, USA) was adopted to construct binary vectors harboring *AtICE1* and *OsICE1* and the details of the primers used for cloning are provided in the Table S1. The *OsICE1* and *AtICE1* genes were cloned into pK7WG2.0 gateway binary vector (www.psb.ugent.be) under the control of *CaMV35S* promoter. These constructs were verified by sequencing and the resulting binary vectors were mobilized into *Agrobacterium tumefaciens* strain EHA105 by electroporation (Electroporator 2510, Eppendorf®, Germany).

Transgenic tobacco (*Nicotiana tabacum* cv. KST) over-expressing *AtICE1* and *OsICE1* were generated by *A. tumefaciens* mediated transformation approach using leaf discs

as explants (Horsch et al. 1985). Leaf discs were co-cultivated with *Agrobacterium* harbouring recombinant binary vector on MS media supplemented with 6-benzylaminopurine (2.25 mg/L) and naphthalene acetic acid (0.1 mg/L) for regeneration. The regenerated shootlets were rooted on MS media supplemented with indole butyric acid (2 mg/L). The rooted plantlets were hardened, transplanted to pots filled with potting mixture and grown under control conditions in transgenic containment facility. Positive transformants were selected by incubating transgenic (T_0 generation) seeds on MS media supplemented with kanamycin (100 mg/L) for a week. The putative transgenic lines with good root growth and better phenotype were selected and progressed to next generation.

Seeds of the T_0 transformants were germinated on MS media supplemented with kanamycin (100 mg/L) to select putative transgenic plants. The putative transgenic plants were selected based on PCR and RT-PCR. In T_2 generation, three independent transgenic lines over-expressing both *AtICE1* (denoted as *AtICE1-1*, *2*, *3*) and *OsICE1* (denoted as *OsICE1-1*, *2* and *3*) with significant transgene expression and normal phenotype were selected and advanced to T_3 generation for all the experiments.

RNA isolation and gene expression analysis

Total RNA was isolated by phenol-choloform method (Sambrook and Russell 2001) and was used to prepare cDNA using oligo(*d*T) primer and M-MuLV reverse transcriptase (MBI Fermentas) according to the manufacture's protocol. RT-PCR was carried out under standard PCR conditions using gene specific primers (Table S1). Real-time quantitative RT-PCR (qRT-PCR) was performed in reaction mixture containing SYBR green (DyNAmo SYBR-Green qPCR Kit FiNNZYMES, Finland) in a real-time PCR machine (MJ Research, USA & MJ Bioworks, Inc.). For normalization, expression of a housekeeping gene actin was used. Relative transcript levels were calculated using comparative C_t method (Pfaffl 2001).

For expression profiling of *ICE1* target genes in tobacco, leaf samples were collected from the WT and transgenic tobacco plants subjected to dehydration stress for 20 d. Total RNA was extracted and cDNA was synthesized as mentioned above. Using the cDNA as template, RT-PCR was performed under standardized conditions using target gene specific primers. The list of genes along with their locus ID, details of primers used are given in the Table S1.

Characterization of tobacco transgenic plants

Seedling level assay

Transgenic tobacco (T_3 generation) plants over-expressing *AtICE1* and *OsICE1* under constitutive promoter (CaMV35S) were used for assessing the role of *ICE1* in abiotic stress tolerance. Seeds were surface sterilized and inoculated on MS media supplemented with NaCl (300 mM) and polyethylene glycol (PEG)-8000 (25 % *w*/*v*). The seedlings were allowed to grow for 10 d and stress effect was analyzed by recording root and shoot growth and also estimating chlorophyll stability index (CSI) (Beltagi 2008). To estimate the CSI, leaf material from the seedlings exposed to different abiotic stresses were washed in distilled water and total chlorophyll was extracted from leaves in 80 % (*v*/*v*) acetone: Dimethyl sulfoxide (1:1 *v*/*v*) mixture. The absorbance of the extract was recorded at 663 and 645 nm using UV-Visible spectrophotometer (UV-Vis, Shimadzu, Japan Model DU800). Total chlorophyll expressed as mg/g of fresh weight (Hiscox and Israelstam 1979) and CSI was calculated using the following formula.

$$\text{CSI}\ (\%) = \{(\text{total chlorophyll before stress} - \text{total chlorophyll under stress}) / (\text{total chlorophyll under stress})\} \times 100$$

Imposition of drought at whole plant level

A gravimetric approach was followed to mimic drought stress as described earlier (Karaba et al. 2007). For this, a known amount of soil was filled in pots and drenched with water. The soil was then allowed to attain saturation (100 % field capacity, FC) and selected transgenic and WT seedlings were transplanted in these pots. All the pots were maintained at 100 % FC for 30 d. Later, one set of plants were maintained at 100 % FC (control) while the other sets were allowed to reach 40 % FC gradually (water stressed) by controlled irrigation.

Effects of moisture deficit condition on physiological processes were quantified as cell viability and cell membrane stability (CMS). Cell viability was estimated by 2,3, 5-triphenyl-tetrazoliumchloride (TTC) reduction test, as described by Towill and Mazur (1975). Electrolyte leakage that reflects loss of membrane integrity was estimated by recording electrical conductivity (EC) using EC-TDS analyzer (ELICO-CM183). Initial electrolytes leaked were recorded (I), subsequently, leaf segments were boiled for 30 min and allowed to cool down and final reading (F) was recorded. Similarly, the leakage was also measured for leaf discs without any stress treatment (control, initial reading (CI) and final reading (CF)) (Tripathy et al. 2000). The CMS was calculated using the following formula.

$$CMS = \{[1-(\mathrm{I}/\mathrm{F})]/[1-(CI/CF)]\}*100$$

Ethylene-induced senescence assay

Leaf discs collected from three weeks old transgenic and WT tobacco plants were surface sterilized and incubated in ethrel solution (1200 ppm) in dark up to 42 h to induce senescence. After 42 h of incubation, total chlorophyll was quantified and CSI was calculated (Beltagi 2008).

Salinity and dehydration stress assay

From three weeks old plants, leaf discs were collected, surface sterilized, and incubated in NaCl (300 mM) or MV (7 μM) for 42 h. Similarly, to induce dehydration stress leaf discs were floated on PEG 8000 (25 % *w/v*) for 42 h and then CMS was estimated.

Statistical analysis

The data generated in all the physiological assays was analyzed by student's t-test (two tailed) to compare statistical differences between WT and transgenic plants.

Results

Evaluation of *Arabidopsis* transgenic plants over-expressing *AtICE1*

To examine the role of *ICE1* in abiotic stress tolerance, *Arabidopsis* transgenic plants over-expressing *ICE1* (Chinnusamy et al. 2003) were used. Exposure to salinity (NaCl, 100 mM) for seven days induced tip necrosis/blackening in WT plants whereas *ICE1* transgenic plants were devoid of such symptoms (Fig. 1a). *ICE1* overexpressors showed lower levels of chlorophyll degradation (Fig. 1b) with significantly higher biomass than WT (Fig. 1c) after prolonged NaCl stress of 20 d. MV-induced oxidative stress caused albino phenotype in WT seedlings after seven days, whereas such phenotypes were not noticed in *ICE1* transgenic

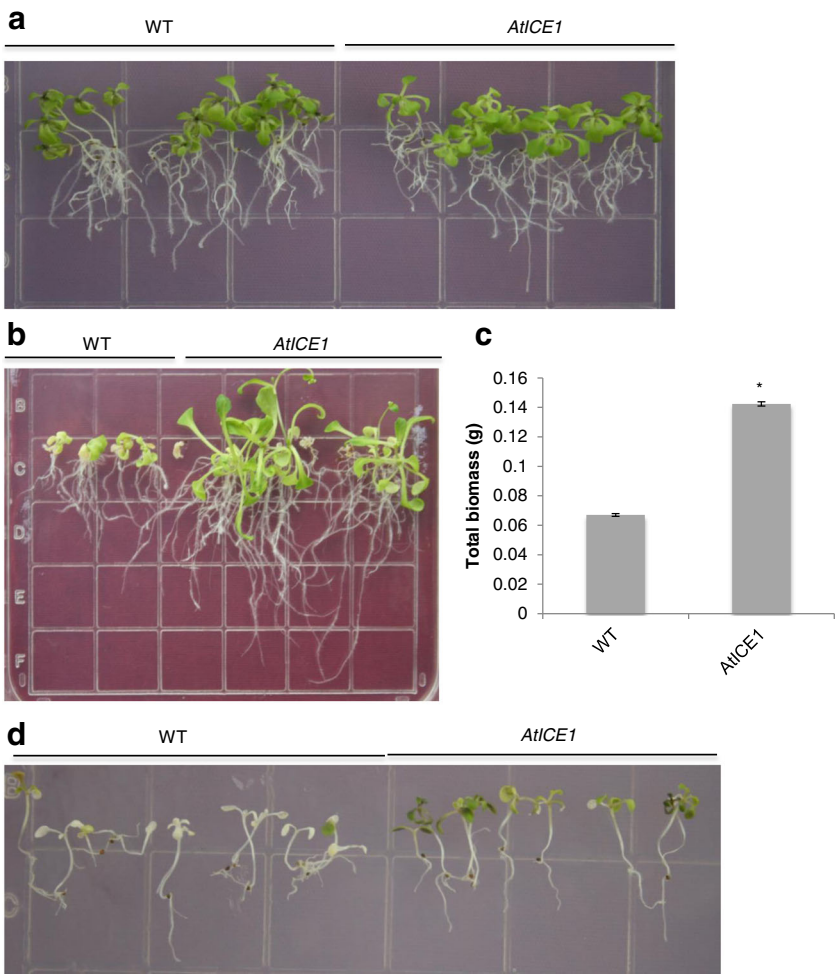

Fig. 1 Response of wild-type (WT) and ICE1 over-expressing *Arabidopsis* seedlings to salt and oxidative stresses. **a** and **b** Phenotypic appearance of seedlings grown on MS agar medium supplemented with 100 mM NaCl for 7 and 20 d respectively. **c** Biomass of *Arabidopsis* transgenics expressing ICE1 after 20 d of NaCl induced stress. **d** Effect of methyl viologen (MV; 5 μM) induced oxidative stress on performance of transgenics and WT after 7 d

plants (Fig. 1d). These results indicated that the *ICE1* is involved in salt and oxidative stress tolerance in *Arabidopsis*.

Generation of transgenic tobacco plants expressing *AtICE1* and *OsICE1* and their evaluation

To further evaluate the role of *ICE1*, we developed transgenic tobacco constitutively over-expressing *AtICE1* and *OsICE*1 (Fig. S1a). We confirmed the expression of transgenes by semi-quantitative and quantitative-RT-PCR analysis in leaf tissue of tobacco transgenic plants (Fig. S1b and S1c). Seedlings of the selected transgenic lines exposed to either NaCl (300 mM) or PEG-8000 (25 % *w*/*v*) stress showed better phenotype than untransformed WT plants 10 d post stress imposition (Figs. 2a and 3a). Transgenic lines showed higher CSI (Figs. 2b and 3b), better root (Figs. 2c and 3c) and shoot growth (Fig. 3d) than WT plants under these conditions, although under NaCl stress there was no significant difference in shoot growth (Fig. 2d). Further, we evaluated drought stress tolerance of plants grown in pots by controlled irrigation. After 20 d of moisture deficit condition, transgenic plants exhibited significantly higher cell viability as reflected by percent TTC reduction (Fig. 4a) and CMS than WT plants (Fig. 4b).

ICE1 retards ethylene and stress-induced leaf senescence

Many abiotic stresses such as oxidative stress induces leaf senescence (Sedigheh et al. 2011). To determine whether *ICE1* over-expression alters this response, leaf discs of WT and *ICE1* transgenics were exposed to ethylene for 42 h. As a result, chlorosis was significant in WT than that of *ICE1* expressing transgenic lines, suggesting that *ICE1* delays ethylene induced leaf senescence. CSI was also significantly higher in transgenic lines as compared to WT treated with ethrel (Fig.

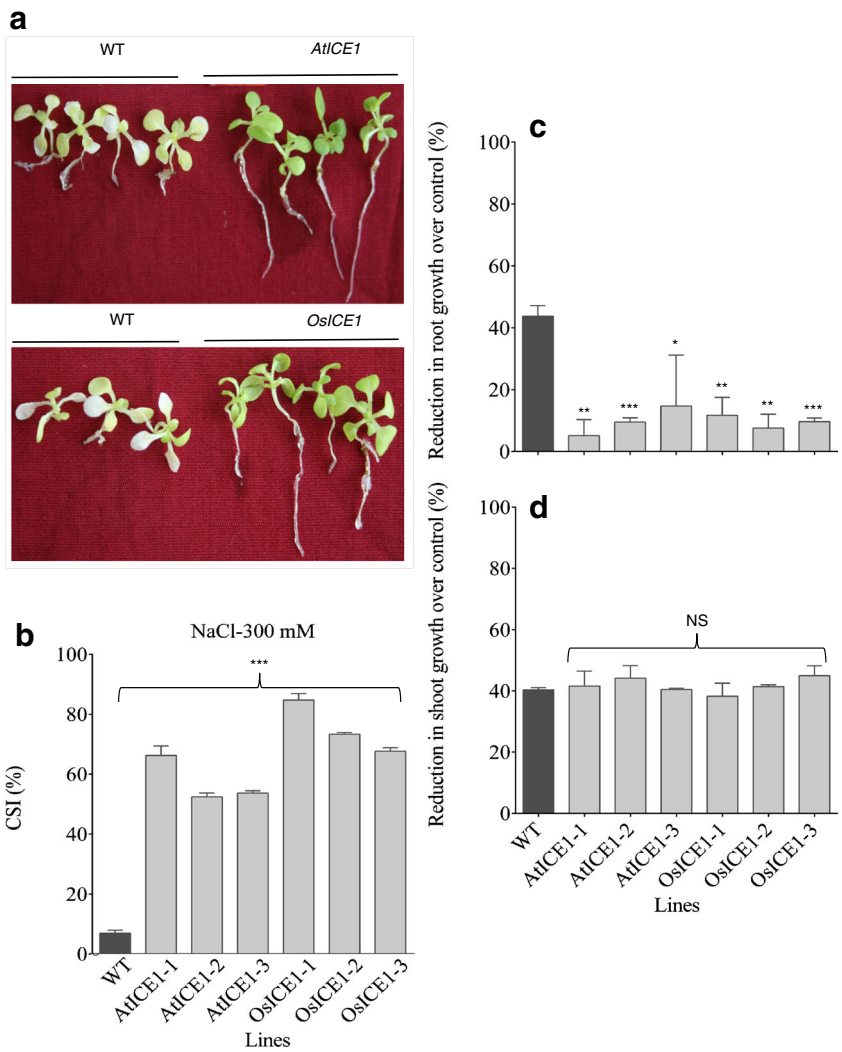

Fig. 2 Response of transgenic tobacco plants over-expressing *AtICE1* and *OsICE1* to salt stress. **a** Seedling growth, **b** CSI and **c** & **d** percent reduction in root and shoot growth of WT and transgenic seedlings, respectively, grown on MS medium supplemented with NaCl (300 mM) for 10 d are presented. Data shown are mean of three replicates, error bars represents standard error and asterisks indicate the significance between WT and ICE1 over-expressed tobacco plants, as described by Student's *t* test: $*P < 0.05$, $**P < 0.005$ and $***P < 0.0001$

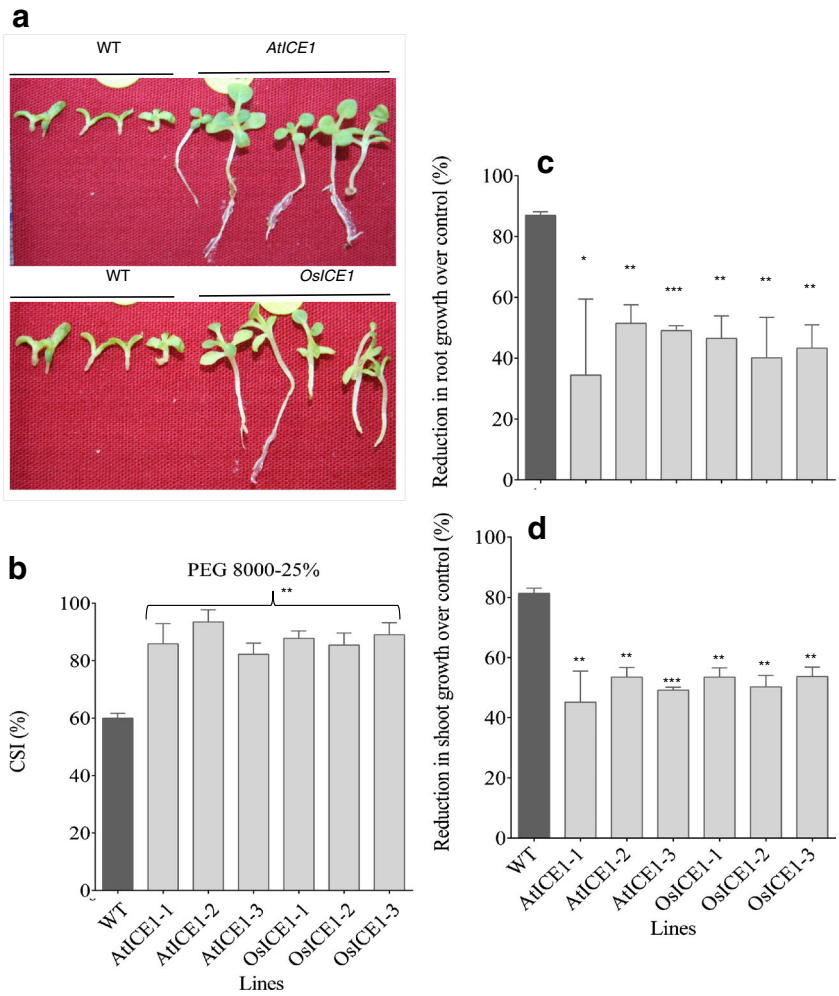

Fig. 3 Response of transgenic tobacco plants over-expressing *AtICE1* and *OsICE1* to PEG induced dehydration stress. **a** Seedling growth, **b** CSI and **c** & **d** percent reduction in root and shoot growth of WT and transgenic seedlings, respectively, grown on MS medium supplemented with PEG 8000 (25 %) for 10 d are presented. Data shown are mean of three replicates, error bars represents standard error and asterisks indicate the significance between WT and ICE1 over-expressed tobacco plants, as described by Student's *t* test: $*P < 0.05$, $**P < 0.005$ and $***P < 0.0001$

S2a). Further, to examine the role of *ICE1* in imparting cellular tolerance under different abiotic stresses, leaf tissue was exposed to NaCl (300 mM), PEG-8000 (25 %) and MV (7 µM). *ICE1* over-expressors retained significantly high CMS as compared to WT under these stresses (Fig. S2b, c and d).

ICE1 over-expression enhanced the expression of stress responsive genes

To study the effect of *ICE1* over-expression on the expression of senescence associated and stress responsive genes, we examined the expression pattern of a few selected target genes of *ICE1* by semi-quantitative RT-PCR. *ICE1* over-expression enhanced the expression of stress responsive genes such as *HSF1* (TC5803), *EREBP3* (TC9256), *ERD10* (At1g20450), *COR47* (TC4052) and *HVA22* (TC5661), where as the expression levels of *WRKY6* (TC3961) was relatively less in transgenic lines as compared to WT (Fig. S3).

Discussion

Over-expression of several *ICE1* homologs including *AtICE1* and *AtICE2* (Chinnusamy et al. 2003; Fursova et al. 2009), *OsICE1* (Xiang et al. 2008; Liu et al. 2010), *TaICE141* and *TaICE187* (Badawi et al. 2008), *CsICE1* (Wang et al. 2012), *MdClbHLH1* (Feng et al. 2012) and *EcaICE1* (Lin et al. 2014) have been shown to enhance cold tolerance in plants. The roles of bHLH and ICE1 homologs have also been extended in other abiotic stresses like drought, osmoticum and salinity. Many ICE1 homologs such as *OsbHLH001* and *OsbHLH002* (Li et al. 2006), *OrbHLH2* (Zhou et al. 2009), *CbICE53* (Zhou et al. 2012) and *CdICE1* (Chen et al. 2012) can impart salinity and osmotic stress tolerance. *OrICE1* has been shown to have role only under salt and osmoticum but not under cold (Zhou et al. 2009), *CdICE1* showed tolerance to cold, salinity and drought (Chen et al. 2012). In a recent study, under cold stress, more complex regulation of ICE1 by OPEN STOMATA 1 (OST1) has been reported (Ding et al. 2015). OST1 phosphorylates ICE1, which stabilizes the TF and promotes its transcriptional activity. Moreover, OST1 interferes with the

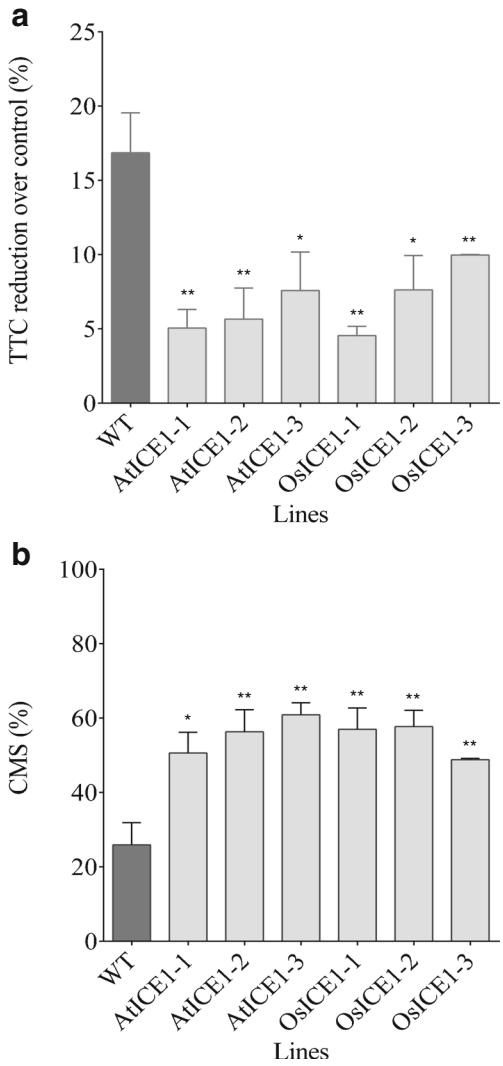

Fig. 4 Response of transgenic tobacco plants over-expressing *AtICE1* and *OsICE1* to drought. WT and transgenic plants were grown in pots for 30 d under normal condition and subsequently, irrigation was withheld for 20 d to impose drought. **a** Percent TTC reduction over control and **b** CMS during stress was used to assess the drought tolerance of WT and ICE1 transgenics. Data shown are mean of three replicates, error bars represents standard error and asterisks indicate the significance between WT and *ICE1* overexpressed tobacco plants, as described by Student's *t* test: $*P < 0.05$, $**P < 0.005$ and $***P < 0.0001$

interaction between HOS1 and ICE1, which also contributes to ICE1 stability and suppresses HOS1-mediated ICE1 degradation.

To explore the possible regulation of ICE1 expression in different plants, we analyzed upstream regulatory region (UTR) of *AtICE1* and *OsICE1* using STIF2db (http://caps.ncbs.res.in/stifdb2). The results indicated that *AtICE1* has 4Myb_box5_myb, 1Myb_box1_MYB, 6HSE1_HSF and 1WRKY TF binding regions whereas the *OsICE1* has no specific stress TF binding sites predicted in the 1000 bp UTR and upstream region. This suggests that the protein probably, is regulated differentially in different species. From this view, we selected *ICE1* gene from both *Arabidopsis* (*AtICE1*) and rice (*OsICE1*) for examining its role in cold sensitive model plant tobacco. Independent transgenic lines expressing these genes exhibited typical phenotype of tolerance to different abiotic stresses as evidenced by excised leaf disc, seedling and whole plant assays.

ICE1 protein has been shown to play an important role in cold acclimation since its discovery (Chinnusamy et al. 2003), by regulating expression of *COR* genes through CBF3 (Chinnusamy et al. 2006). In *Arabidopsis* Chinnusamy et al. (2003) showed that *ICE1* transcripts are constitutively expressed in all the tissues and the levels were high under different abiotic stresses like cold, NaCl and ABA. However, under dehydration stress, the *ICE1* induction was not in high levels as compared to other stresses (Chinnusamy et al. 2003). Further, GFP-ICE1 fusion protein was localized to nucleus independent of stress (cold or warm) conditions. We, in this study, attempted to demonstrate additional role of *ICE1* in abiotic stress acclimation. At seedling stage, *A. thaliana* transgenic lines over-expressing *AtICE1* exhibited normal phenotype under NaCl-and MV-induced stresses with significantly higher biomass in prolonged NaCl stress than the WT (Fig. 1). Similarly, *ICE1* expressing tobacco transgenic seedlings exposed to NaCl and PEG-8000 induced stresses maintained better growth and development (Figs. 2 and 3). The stress tolerant phenotype of both *A. thaliana* and tobacco transgenic plants over-expressing *ICE1* is probably due to the activation of diverse cellular tolerance pathways.

Significantly higher membrane integrity, chlorophyll stability and cell viability observed under stressful condition (Figs. 4 and S2) in ICE1 over-expressors suggest that the TF acts as upstream regulator coordinating multiple cellular tolerance mechanisms. This is evident from our downstream target gene expression study. We examined the expression of a few *ICE1* target genes containing MYB, WRKY and HSE cis elements in their promoters. The downstream targets such as *HVA22*, *ERD10*, *COR47*, *HSF1* and *WRKY6* were selected using STIFdb analysis (Sundar et al. 2008). The *AtICE1* and *OsICE1* over-expressers showed up-regulation of *HVA22*, *ERD10* (dehydrin), and *COR47* (dehydrin) (Fig. S3). These downstream genes, which were induced by ICE1, have been demonstrated to play a role in abiotic stress acclimation (Zhou et al. 2009). We also observed up-regulation of *HSF1* transcripts in transgenic lines, which is known to be essential for stress acclimation (McMillan et al. 1998). Similarly, in *ICE1* over-expressers, we noticed reduced expression of *WRKY6*, a gene having role in senescence-associated processes (Robatzek and Somssich 2002). Further, *EREBP3* a negative regulator of senescence (Ma et al. 2009) was found to be up-regulated in *ICE1* over-expressers. This altered regulation of *WRKY6* and *EREBP3* relates the ethylene-induced delayed leaf senescence in transgenic plants. Taken together, the

results indicate that constitutive expression of *ICE1* can improve cellular tolerance to multiple abiotic stresses through diverse mechanisms. Further work towards identifying downstream targets of ICE1 and their interactions would be useful for targeted crop improvement towards abiotic stress tolerance.

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Compliance with ethical standards We declared that no competing interests exist (financial or non-financial). In this study there is no involvement of any human participants and/or animals.

Conflict of interest The authors declare that they have no conflict of interest.

References

Agarwal M, Hao Y, Kapoor A, Dong CH, Fujii H, Zheng X, Zhu JK (2006) A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. J Biol Chem 281:37636–37645

Badawi M, Reddy YV, Agharbaoui Z, Tominaga Y, Danyluk J, Sarhan F, Houde M (2008) Structure and functional analysis of wheat ICE (Inducer of CBF Expression) genes. Plant Cell Physiol l49: 1237–1249

Beltagi MS (2008) Molecular responses of Bt transgenic corn (*Zea mays* L.) plans to salt (NaCl) stress. Aust J Crop Sci 2:57–63

Boyer JS (1982) Plant productivity and environment. Science 218: 443–448

Chen L, Chen Y, Jiang J, Chen S, Chen F, Guan Z, Fang W (2012) The constitutive expression of *Chrysanthemum dichrum* ICE1 in *Chrysanthemum grandiflorum* improves the level of low temperature, salinity and drought tolerance. Plant Cell Rep 31:1747–1758

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcription and freezing tolerance in *Arabidopsis*. Genes Dev 17:1043–1054

Chinnusamy V, Zhu J, Zhu JK (2006) Gene regulation during cold acclimation in plants. Physiol Plant 126:52–61

Chinnusamy V, Zhu JK, Sunkar R (2010) Gene regulation during cold stress acclimation in plants. Methods Mol Biol 639:39–55

Ding Y, Li H, Zhang X, Xie Q, Gong Z, Yan S (2015) OST1 kinase modulates freezing tolerance by enhancing ICE1 stability in Arabidopsis. Dev Cell 32:278–228

Dong CH, Agarwal M, Zhang Y, Xie Q, Zhu JK (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. Proc Natl Acad Sci U S A 103:8281–8286

Feng XM, Zhao Q, Zhao LL, Qiao Y, Xie XB, Li HF, Yao YX, You CX, Hao YJ (2012) The cold-induced basic helix-loop-helix transcription factor gene MdCIbHLH1 encodes an ICE-like protein in apple. BMC Plant Biol 12:22

Fursova OV, Pogorelko GV, Tarasov VA (2009) Identification of ICE2, a gene involved in cold acclimation which determines freezing tolerance in *Arabidopsis thaliana*. Gene 429:98–103

Hiscox JD, Israelstam GF (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot 57:1332–1334

Horsch RB, Fry JE, Hoffmann N, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Karaba A, Dixit S, Greco R, Aharoni A, Trijatmiko KR, Marsch-Martinez N, Krishnan A, Nataraja KN, Udayakumar M, Pereira A (2007) Improvement of water use efficiency in rice by expression of HARDY, an *Arabidopsis* drought and salt tolerance gene. Proc Natl Acad Sci U S A 104:15270–15275

Knight H, Zarka DG, Okamoto H, Thomashow MF, Knight MR (2004) Abscisic acid induces CBF gene transcription and subsequent induction of cold-regulated genes via the CRT promoter element. Plant Physiol 135:1710–1717

Lata C, Prasad M (2011) Role of DREBs in regulation of abiotic stress responses in plants. J Exp Bot 62:4731–4748

Li X, Duan X, Jiang H, Sun Y, Tang Y, Yuan Z, Guo J (2006) Genome-wide analysis of basic/helix-loop-helix transcription factor family in Rice and *Arabidopsis*. Plant Cell 141:1167–1184

Lin Y, Zheng H, Zhang Q, Liu C, Zhang Z (2014) Functional profiling of EcaICE1 transcription factor gene from *Eucalyptus camaldulens is* involved in cold response in tobacco plants. J Plant Biochem Biotechnol 23:141–150

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10:1391–1406

Liu L, Duan L, Zhang J, Zhang Z, Mi G, Ren H (2010) Cucumber (*Cucumis sativus* L.) over-expressing cold-induced transcriptome regulator ICE1 exhibits changed morphological characters and enhances chilling tolerance. Sci Hortic 124:29–33

Ma T, Chen R, Yu R, Zeng H, Zhang D (2009) Differential global genomic changes in rice root in response to low-, middle-, and high-osmotic stresses. Acta Physiol Plant 31:773–785

McMillan DR, Xiao X, Shao L, Graves K, Benjamin IJ (1998) Targeted disruption of heat Shock transcription factor 1 abolishes thermos tolerance and protection against heat-inducible apoptosis. J Biol Chem 273:7523–7528

Miura K, Jin JB, Lee J, Yoo CY, Stirm V, Miura T, Ashworth EN, Bressan RA, Yun DJ, Hasegawa PM (2007) SIZ1-mediated sumoylation of ICE1 controls CBF3/DREB1A expression and freezing tolerance in *Arabidopsis*. Plant Cell 19:1403–1414

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–479

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45

Pruthvi V, Rama N, Nataraja KN (2014) Simultaneous expression of abiotic stress responsive transcription factors, *AtDREB2A*, *AtHB7* and *AtABF3* improves salinity and drought tolerance in peanut (*Arachis hypogaea* L). PLoS One 9:e111152

Robatzek S, Somssich IE (2002) Targets of *At*WRKY6 regulation during plant senescence and pathogen defense. Genes Dev 16:1139–1149

Sambrook J, Russell DW (2001) Molecular cloning: A laboratory manual 3rd edition. Cold Spring Harbor, Laboratory Press

Sedigheh GH, Mortazavian M, Norouzian D, Atyabi M, Akbarzadeh A, Hasanpoor K, Ghorbani M (2011) Oxidative stress and leaf senescence. BMC Res Notes 4:477

Sundar AS, Varghese SM, Shameer K, Karaba NN, Udayakumar M, Sowdhamini R (2008) STIF: Identification of stress-upregulated transcription factor binding sites in *Arabidopsis thaliana*. Bioinformation 2:431–437

Towill LE, Mazur P (1975) Studies on the reduction of 2,3,5-triphenyl tetrazolium chloride as a viability assay for plant tissue cultures. Can J Bot 53:1097–1102

Tripathy JN, Zhang J, Robin S, Nguyen Thuy T, Nguyen HT (2000) QTLs for cell-membrane stability mapped in rice (*Oryza sativa L.*) under drought stress. Theor Appl Genet 100:1197–1202

Wang Y, Jiang CJ, Li YY, Wei CL, Deng WW (2012) CsICE1 and CsCBF1: two transcription factors involved in cold responses in *Camellia sinensis*. Plant Cell Rep 31:27–34

Xiang DJ, Hu XY, Zhang Y, Yin KD (2008) Over-expression of ICE1 gene in transgenic rice improves cold tolerance. Rice Sci 15: 173–178

Yang S, Vanderbeld B, Wan J, Huang Y (2010) Narrowing down the targets: towards successful genetic engineering of drought-tolerant crops. Mol Plant 3:469–490

Zhou J, Li F, Wang J, Ma Y (2009) Basic helix-loop-helix transcription factor from wild rice (*OrbHLH2*) improves tolerance to salt- and osmotic stress in *Arabidopsis*. J Plant Physiol 166:1–11

Zhou M, Wu L, Liang J, Shen C, Lin J (2012) Cold-induced modulation of *CbICE53* gene activates endogenous genes to enhance acclimation in transgenic tobacco. Mol Breeding 30:p1611