Increased Drought Tolerance through the Suppression of *ESKMO1* Gene and Overexpression of CBF-Related Genes in Arabidopsis

Fuhui Xu1, Zhixue Liu1, Hongyan Xie1, Jian Zhu1, Juren Zhang3, Josef Kraus2, Tasja Blaschnig2, Reinhard Nehls2, Hong Wang1,2*

1 School of Life Sciences and Technology, Tongji University, Shanghai, China, 2 KWS SAAT AG, Einbeck, Germany, 3 School of Life Science, Shandong University, Shandong, China

**Abstract**

Improved drought tolerance is always a highly desired trait for agricultural plants. Significantly increased drought tolerance in *Arabidopsis thaliana* (Columbia-0) has been achieved in our work through the suppression of *ESKMO1* (*ESK1*) gene expression with small-interfering RNA (siRNA) and overexpression of CBF genes with constitutive gene expression. *ESK1* has been identified as a gene linked to normal development of the plant vascular system, which is assumed directly related to plant drought response. By using siRNA that specifically targets *ESK1*, the gene expression has been reduced and drought tolerance of the plant has been enhanced dramatically in the work. However, the plant response to external abscisic acid application has not been changed. *ICE1, CBF1*, and *CBF3* are genes involved in a well-characterized plant stress response pathway, overexpression of them in the plant has demonstrated capable to increase drought tolerance. By overexpression of these genes combining together with suppression of *ESK1* gene, the significant increase of plant drought tolerance has been achieved in comparison to single gene manipulation, although the effect is not in an additive way. Accompanying the increase of drought tolerance via suppression of *ESK1* gene expression, the negative effect has been observed in seeds yield of transgenic plants in normal watering conditions comparing with wide type plant.

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* Email: hong.wang@kws.com

**Introduction**

Drought stress is a major limiting factor for crop production worldwide [1]. In 2012, a severe drought in the United States caused heavy losses in crop production, especially in corn, and farmers produced less than three-fourths of the corn that the U.S. Department of Agriculture anticipated [2]. In China, around 20 million hectares of land are at risk of drought each year [3]. Globally, estimated crop losses due to water limitation exceed $10 billion annually [4]. Improving yield under drought, therefore, is a continuous challenge for agriculture, especially for modern breeding.

The development of modern plant biotechnology provides new hope for generating crops with increased drought tolerance. Understanding the response of plants to drought stress is the first step for development of stress tolerance plant through plant biotechnology. Gene expression experiments have identified several hundred genes that are either induced or repressed during drought. Some of those genes encode proteins that play important role in protecting cells from dehydration, such as the enzymes required for biosynthesis of various osmoprotectants, late-embryo-genesis–abundant (LEA) proteins, antifreeze proteins, chaperones, and detoxification enzymes [5]. Some others are responsible for gene products including transcription factors, protein kinases, and enzymes involved in phosphoinositide metabolism. C-repeat/dehydration-responsive element binding factors (CBFs) are AP2/ERF-type transcription factors, which make up a critical gene cluster of the second group. During in the stress condition, CBF genes are rapidly induced in response to abiotic stress, such as dehydration and cold [6,7]. The CBF proteins in turn activate expression of a set of target effector genes by binding to a core sequence in their promoter, C-repeat (CRT) / dehydration response element (DRE) [8,9,10,11]. CBF genes appear to be ubiquitous in plant species and almost always present as a gene family [12,13]. In *Arabidopsis*, the three characterized CBF genes are *CBF1, CBF2*, and *CBF3*, which are organized in tandem on chromosome 4 [14]. *CBF1* and *CBF3* are positive regulators whereas *CBF2* has a negative regulatory role [15]. CBF transcription factor genes are induced by the constitutively expressed inducer of CBF expression (*ICE1*) by binding to the CBF promoter [16,17]. The *ICE1-CBF* cold response pathway is
conserved in diverse plant species [14,17,18]. Constitutive overexpression of CBF transcription factors in transgenic plants has increased the plant tolerance to freezing, salt, and drought stresses [8,19,20,21,22,23]. This functional conservation has suggested the ICE1-CBF genes are important targets for crop improvement for drought tolerance through genetic engineering [24].

ESK1 is a newly discovered member of the second group. It was initially identified as conferring freezing tolerance; a significantly high proline content accumulates in esk1 mutants [25]. The gene product of ESK1 belongs to an uncharacterized plant-specific protein family containing 48 members [26]. Bioinformatics analysis of genes whose expression modified by the esk1 mutant showed that a large number of genes were previously reported linking to plant response to salt, osmotic stress, and the stress hormone abscisic acid (ABA) [25,26]. Later work showed that the mutant has a clear advantage in response to drought and salt stress: In standard and drought conditions, transpiration rate of mutant is lower than in wild type (WT) [27]. A biologically relevant parameter is the water required per biomass unit, and with this measure, the esk1 mutants clearly have shown a higher water use efficiency and photosynthetic rate compared to WT [27]. This higher water use efficiency was independent of stomata closure through ABA biosynthesis. Measurement of root hydraulic conductivity suggests that the esk1 vegetative apparatus suffers water deficit because of a defect in water transport system [27,28]. ESK1 promoter-driven reporter gene expression has been observed in xylem and fibers, the vascular tissue which is responsible for the transport of water and mineral nutrients from the soil to the shoots, via the roots. Moreover, in cross sections of hypocotyls, roots, and stems, collapsed xylem vessel has been observed in esk1 mutant [28]. The ESK1 gene, therefore, was inferred to play a major role in whole plant water economy, ESK1 has homologues in numerous species, and it is reasonable to hypothesize that manipulation of ESK1 in crops could improve water use efficiency.

With an understanding of the molecular mechanism, several gene manipulation approaches have been employed to increase plant drought tolerance. The manipulated genes include those encoding enzymes required for the biosynthesis of various osmoprotectants or enzymes for modifying membrane lipids, LEA protein, and detoxification enzyme [29,30,31]. To date, the CBF genes are the most explored genes for improving crop stress tolerance because of the ability of this transcription factor to regulate an entire set of genes in a stress-response pathway [8,32,33]. When rice OsDREB1A [34] or corn ZmDREB1A is constitutively overexpressed in Arabidopsis, the downstream target genes regulated by the Arabidopsis DREB1 (e.g., RD29A) are induced, resulting in desiccation tolerance under 15% humidity [35]. In another study, 35S: CBF1 transgenic tomato plants are more resistant to water-deficit stress by showing less plant wilting and leaf curling than WT controls after 21-d water withdrawal in the same pot [36]. Constitutive overexpression of two wheat CBF factors in barley substantially improves survival under severe drought or cold. In addition, expression of DREB2 factors in wheat and barley under the control of drought-inducible promoters allows for normal development, together with significantly improved survival under severe drought [37].

In spite of the extensive evaluation of CBF factors, only a few studies have shown a clear improvement in drought tolerance in crops under field conditions [38]. Considering the complexity of the plant stress response, it has been assumed that better drought tolerance might be obtained if multiple genes involved in different stress response pathways could be manipulated together through molecular stacking. In this work, we selected ICE1 as well as the CBF1 and CBF3 genes involved in one stress-activated pathway and the ESK1 gene from another stress-regulating pathway as targets of gene manipulation. Here, ICE1 or CBF1 and CBF3 genes were overexpressed under the control of a constitutive promoter whereas the ESK1 gene was suppressed with siRNA technique. Using gene stacking, we combined the two expression cassettes into one transformation vector for Agrobacterium-mediated plant transformation. The results obtained by testing the concept in the model plant Arabidopsis showed a significant increase in drought tolerance compared to non-transgenics, indicating that multiple gene manipulation might be a promising strategy for improving stress response and especially drought tolerance in plants.

Results

Manipulation of target gene expression through knock-down and overexpression

To knock down gene expression, a siRNA targeting specifically the ESK1 gene was designed, and to overexpress the desired ICE1 or CBF gene, the 35S promoter was placed in front of the gene for constitutive expression. The gene suppression and overexpression cassettes were integrated into the Arabidopsis genome, either alone or combined, via the floral dipping method. In analysis of gene expression by qRT-PCR, the plant transformed with siRNA cassette that suppresses ESK1 showed clear knock-down of ESK1 expression. Although the level of suppression varied from line to line, most plants showed about or more than 50% reduction in gene expression (Fig. 1A). In comparison, the transgenic lines derived via using only the pGPTV-ESK1 vector showed a better suppression of ESK1 gene expression than those obtained through use of the vector with the stacked overexpression and suppression cassettes (Figs. 1A, C, D).

Analysis of either ICE1 or CBF overexpression showed the transgenic lines with the relevant transgene cassette had significantly higher ICE1 or CBF expression compared to WT (Figs. 1B, E). For further evaluation of the concept, the best-performing plants based on qRT-PCR results were selected and their T2 seeds produced following the protocol described in the Methods part.

The response of transgenic Arabidopsis to osmotic stress in vitro

A distinct difference emerged in in vitro stress response between transgenic and WT plants. When growing on medium without any applied external stress, the majority of transgenic lines showed an almost identical phenotype to WT plants. However, when growing on medium with 30% polyethylene glycol (PEG), the transgenic plants with suppressed ESK1 had much better root system growth than WT plants: The main root was longer, and the number of lateral roots was greater (Fig. 2). Leaf growth also differed between the transgenic and WT plants although not as much as the root system: All transgenic lines had eight leaves after 18 days of growth on medium with PEG whereas WT had only six. 15 ESK1 transgenic lines were tested and the result of response to PEG was similar (data not shown).

Compared to the transgenic lines with the suppressed ESK1 alone, the plants obtained through using stacking vector that suppress ESK1 and overexpress either ICE1 (14 lines) or CBF (3 lines) presented a similar performance on medium with PEG. After 14 days of growth with PEG, more and larger leaves were observed in transgenic lines compared to WT (Fig. 2). The growth
Figure 1. qRT-PCR results show ESK1, ICE1, CBF1, and CBF3 expression in different transgenic lines. (A) ESK1 was tested in two-week-old ESKi transgenic lines. (B) CBF1 and CBF3 were tested in two-week-old CBF transgenic lines. (C) ESK1 and ICE1 were tested in two-week-old eski-ICE1 transgenic lines. (D) ESK1, CBF1 and CBF3 were tested in two-week-old esk2i-CBF transgenic lines. (E) ICE1 was tested in two-week-old ICE1 transgenic lines. Values represent means ± SD (error bar) of three replicates.
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Figure 2. Response of transgenic lines to osmotic stress. Seedlings of different transgenic lines were subjected to osmotic stress: WT, esk1i-3, esk1i-ICE1-7, esk2i-CBF-2, CBF-3, ICE1-3. Four-day-old WT or transgenic seedlings were transferred to 1/2 Murashige and Skoog (MS) medium previously infused with 30% PEG for 14 days. Experiments were repeated at least three times with similar results. At least 30 seedlings per genotype were measured in each replicate.
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of the root system had the same pattern with transgenic lines showing less effect from PEG treatment.

The responses to in vitro osmotic stress observed from transgenic plants with only the overexpression for CBF (5 lines), or ICE1 (4 lines) were also similar to those with ESK1 suppressed (Fig. 2). On normal 1/2 MS medium without PEG, no phenotype difference was observed between transgenics and WT control. On PEG medium, however, most transgenic plants clearly showed better growth than control plants after 14 days. The development of the root system from plants with CBF or ICE1 overexpression was more robust, with a longer main root and more lateral roots. Nevertheless, several lines of ICE1 transgenic plants showed a stress phenotype like that of WT, although the ICE1 expression in those plants was much higher than in WT.

The response of transgenic plants to ABA stress in vitro

Suppression of ESK1 gene expression did not significantly influence the sensitivity of plants to external ABA application. On medium without additional ABA, transgenic plants containing ESK1 suppression cassette appeared almost identical to WT plants in their growth (Fig. 3A). On medium with 20 μM ABA for 14 days, the two still did not differ, and both appeared to suffer the effects of growth on ABA, showing leaves with yellow-brown stress symptoms (Fig. 3B).

The only difference observed between transgenic lines and WT plants was in seed germination time. On medium containing 0.5 μM ABA (Fig. 4B), the transgenic seeds showed delayed germination compared to WT control whereas on medium without ABA (Fig. 4A), the transgenic seeds germinated at the same time as WT.

Similar results were obtained for plant overexpression of CBF or ICE1 in response to ABA stress in vitro (Figs. 3, 4). On medium without ABA, both transgenic and control plants showed similar growth with no abnormal phenotype observed in the majority of plants during the first 18 days of growth.

On medium with 20 μM ABA, both transgenic and WT plants were negatively affected. The elongation of the main root was inhibited, the number of lateral roots was reduced, and the leaves showed yellow-brown stress symptoms. No clear differences between transgenic lines and non-transgenic controls were observed (Fig. 3).

The performance of greenhouse transgenic plants in response to drought

To mimic most completely the interaction of plants and their natural environment, the transgenic plants showing the potential to increase drought tolerance via the in vitro osmotic stress were transferred to greenhouse for evaluation of drought tolerance. The testing protocol was established based on the water-loss rate in greenhouse and the survival rate of plants in the protocol of water withdrawal and re-watering. Plants growing 2 weeks after germination in the described conditions were withdrawn from water for 14 days, so that the soil water content reached around 20%, which is considered a serious drought condition [27]. Following this threshold point, the plants were then fully watered and grown under a normal watering program for two more weeks before survival rates were evaluated.

Non-transgenic plants usually died completely under this protocol; however, transgenic plants obtained by the vector pGPTV-ESKi alone showed dramatic improvement in drought tolerance in contrast to the WT control plant (Fig. 5A). After 14 days without water, the transgenic plants still appeared green although with some level of dryness (Figs. 5A, B). Two weeks after re-watering, many of the transgenic plants had re-gained growth, and the survival rate of the best-performing line esk1i-3 reached 80% (Fig. 5C). In comparison, almost all WT plants became yellow and dry after 14 days without water, seldom regaining growth after water restoration (survival rate 1.25%, Fig. 5C).

Transgenic plants with ICE1 or CBF gene overexpression vector alone also showed increased drought tolerance under greenhouse conditions (Figs. 5B–E). After 14 days without watering, the transgenic plants still remained green and recovered soon after the re-watering; the non-transgenic controls did not recover. Among the transgenic lines with various vectors, the line transformed with the CBF-1 overexpression vector showed relatively better drought tolerance than those transformed with CBF overexpression vector (Figs. 5C, D, E).

The improved drought tolerance test response under greenhouse conditions was also achieved in transgenic plants containing the combined ESKi cassette and overexpression cassette, especially those with the combination of the ESKi and ICE1 cassettes; the highest survival rate was obtained from the line transformed with ESKi-ICE1 stacking vector at 83.25% (Fig. 5C).

To compare the effects of different vectors on improvement of drought tolerance, the transgenic plants were divided into groups according to the vectors used, and the survival rate data was analyzed using t-tests. The results showed that the plants performing best were those transformed with ESKi-ICE1 stacking vector; the lines transformed with the ESKi vector showed the second-best improvement while improvement was weakest with overexpression of the CBF genes (Figs. 5D, E).

The effect of transgenes on seed biomass

To evaluate potential applications to agriculture, we investigated the effect of transgenes on plant biomass, especially seed mass, under greenhouse conditions, either normal growth conditions or drought test conditions.

Under normal growth conditions in the greenhouse, some ESK1 siRNA transgenic plants showed a dwarf phenotype with dark leaves (Fig. 6Aa); a delay in flowering was also observed, but most of the plants, otherwise, looked normal. Measuring the mass of seed harvested under normal greenhouse conditions revealed that the ESK1 siRNA transgenic plants produced less seed than non-transgenic controls. However, upon harvest of the seed from plants that survived the drought test procedure, a clear contrast was evident between the seed yield of transgenic plants and controls, given that most of the non-transgenic plants died of drought. Comparing the seed yield of transgenic plants under drought and normal conditions, a significant reduction was found under drought, with less than 10% of the yield under normal conditions (Fig. 6B).

The impact of transgenes on seed yield showed a similar trend in plants transformed with ICE1 or CBF vector alone and in those transformed with stacking vectors (Fig. 6B). Some dwarf phenotype development was observed during growth under normal conditions whereas in the drought condition, a higher stress tolerance compared to WT was observed, although accompanied by severe seed yield reduction.

Discussion

Plant genetic engineering has presented great potential for improving the drought tolerance of crops, especially since the discovery of CBF genes and their functions in stress tolerance [8,19,20,21,22,23]. ESK1 was recently found to be involved in a stress tolerance pathway that is separate from the CBF pathway [25,26]. The ESK1 pathway originally was identified as being related to plant freezing tolerance, but Bouchabke-Coussa et al.
later observed reduced respiration capacity in the eskl mutant of Arabidopsis, indicating a potential role of ESK1 in drought tolerance. In our experiment, siRNA specifically targeting ESK1 was employed to suppress its expression in Arabidopsis and the role of ESK1 was confirmed in drought tolerance: Transgenic plants with efficient ESK1 expression knock-down showed an obvious increase in osmotic stress tolerance in vitro and little effect on root system development by PEG treatment in vitro (Fig. 2). Further evaluation confirmed that suppression of the ESK1 gene significantly enhanced plant drought tolerance (Fig. 5). In ABA in vitro assay, it has been observed that ESK1 knock-down transgenic plants has the same sensitivity to ABA change as control (Figs. 3B and 4B), which indicates that acquired stress tolerance of the transgenic plant may not be closely linked to the ABA-regulated network.

The ESK1 knock-down plants appeared phenotypically normal comparing to non-transgenic plants when grown for 18 days: Plant size, leaf color, and shape were basically same like WT under normal growth conditions (Fig. 3A). Morphological observation also revealed that the number of stomata in the leaves remained unchanged relative to controls (data not shown). The question that arises is how ESK1 knock-down confers higher tolerance to drought or other stress. One explanation is that the disruption in ESK1 function may block the normal development of the vascular system. Lefebvre et al. [28] observed a kind of collapsed xylem structure in cross sections of hypocotyls, roots, and stems of an Arabidopsis eskl mutant. In the current study, we also observed that the vascular system appeared abnormal in both leaf and stem (data not shown). These observations may explain why the respiration rate of a transgenic plant is lower compared to WT and why drought tolerance is higher than in controls.

Measuring the weight of total seeds showed that knocking down the ESK1 gene can negatively affect seed production under normal growth conditions but that under the drought condition, the yield is much less affected, in contrast to complete loss in the non-transgenic control (Fig. 6B). Linking the yield penalty and the vascular distortion of the ESK1 knock-down plant, we can infer that manipulation of ESK1 gene expression can indeed increase the drought tolerance of plants, but expecting no reduction in biomass is not realistic considering the physical distortion of the vascular structure. Adoption of an inducible promoter or tissue-specific promoter may alleviate some negative consequences, but...
the enhancement of drought tolerance may be compromised at the same time. Therefore, use of ESK1 for stress tolerance should be carefully balanced. One potential may be in improvement of ornamental plants, for which biomass is not important.

Many studies have illustrated the potential of manipulating CBF/DREB genes to confer improved drought tolerance [20,21,37,39]. For example, overexpression of CBF1/DREB1B from Arabidopsis improves tolerance to water-deficit stress in tomato, but few plants clearly show enhanced drought tolerance under natural conditions [36]. We speculate that the complexity of the stress response pathway could be the reason and that modification of a single gene in a complicated pathway might not be sufficient to alter plant drought tolerance dramatically. Gene discovery and functional genomics projects have revealed many mechanisms and gene families that confer improved adaptation to abiotic stresses. These gene families can be manipulated into novel combinations, expressed ectopically, or transferred to species in which they do not naturally occur or vary [40,41,42]. Therefore, we have designed a gene stacking strategy by combining manipulation of the CBF and ESK1 genes, hoping to obtain at least an additive effect on drought tolerance.

The result showed that gene stacking can indeed further improve drought tolerance in Arabidopsis, but an additive effect was not observed. One possibility for the lack of additive effect is that genes stacked in the same transformation vector may not work as efficiently as those in completely independent transformation vectors. qRT-PCR analysis in our work showed that the suppression effect of siRNA targeting ESK1 was less significant when the same vector was used for the stacked RNAi cassette and the overexpression cassette. In the future, the effect of combined gene manipulation will be evaluated through stacking the gene cassette by crossing the transgenic lines with individual vectors.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) and transgenic plants in Col-0 background were grown under long-day conditions (16 h light/8 h dark) with light intensity at 100 μE m⁻² s⁻¹.

For plate-grown plants, Arabidopsis thaliana seeds were surface sterilized with 20% (v/v) bleach and sown on medium containing half-strength Murashige and Skoog (MS) salts [43], 1% sucrose, and 0.8% agar. After stratification for 3 d at 4°C, the plates were kept in a growth incubator under a long-day photoperiod (16 h light, 8 h darkness) at 24°C for 10 d. For transgenic seeds, medium was supplemented with 50 μg/mL kanamycin sulfate.

Gene isolation and binary vector construct

The leaf of 2-week-old Arabidopsis Col-0 was used for DNA and RNA extraction. The Col-0 cDNA sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/). Total DNA was extracted from the samples using the DNeasy Plant Mini Kit (Qiagen, Germany), and total RNA was extracted using the RNeasy Plant Kit (Qiagen, Germany). cDNA was synthesized as described in the Thermo Scientific protocol (p7K1631).
The AtCBF1 (AT4G25490) and AtCBF3 (AT4G25480) were amplified from Arabidopsis Col-0 DNA by PCR. AtCBF1 was amplified with a forward primer containing a BamHI restriction site at the 5’ end (5’-CGGGATCCCTCTGATCAATGAACTCATT-3’) and a reverse primer containing a SacI site at the 3’ end (5’-GCGAGCTCTTAGTAACTCCAAAGCGACA-3’). AtCBF3 was amplified with a forward primer containing an ApaI site at the 5’ end (5’-GGGCCCGATCAATGAACTCATTTTCTGC-3’) and a reverse primer containing a XbaI site at the 3’ end (5’-GCTCTAGATTAATAACTCCATAACGTCGTCG-3’).

The AtICE1 (AT3G26744) and AtESK1 (AT3G55990) cDNA fragments were amplified from Arabidopsis Col-0 RNA by RT-PCR. AtICE1 was amplified with a forward primer containing a XhoI site at the 5’ end (5’-GCCTCGAGGCGATGGGTCTTGACGGAAACAATGGTG-3’) and a reverse primer containing a XbaI site at the 3’ end (5’-GCTCTAGATCAGATCATACCAGCATACCCTGCTGTATCG-3’).

For the RNA interference (RNAi) construct, for a 341-bp specific fragment of ESK1, ek1i, the sense fragment was amplified by PCR using a forward primer (5’-GCCTCGAGTTGCTAGCATGTCTCCTCTT-3’) and a reverse primer (5’-GCGAGCTCATTCACGTGTCAGGTAAAC-3’), as was the antisense fragment (forward primer: 5’-CGCCCGGGATTCCACGTGTCAAAAC-3’; reverse: 5’-CGGTCGACTTGCTAGCATGTCTCCTCTT-3’). For a 284-bp specific fragment of ESK1, ek2i, the sense fragment also was amplified using forward and reverse primers (5’-GCCTCGAGTCAAGTGTGCATTAGACG-3’ and 5’-GCGAGCTCATTCACGTGTCAGGTAAAC-3’, respectively), as was the antisense fragment (forward: 5’-CGGCCCGGGATTCCACGTGTAACAC-3’; reverse: 5’-CGGTCGACTCAAGTGTGCATTAGAGACG-3’). The

Figure 5. Drought responses of WT and transgenic plants. (A) and (B) Drought tolerance assay. 7-d seedlings were transferred to soil for another 1 week, subjected to drought by water withholding for 14 d, and then re-watered for 7 d. WT (Aa and Ac), ek1i-3 (Ab and Ad), ek2i-1 (Ba), ek1i-ICE1-7 (Bb), ek2i-ICE1-6 (Be), ek2i-CBF-2 (Bd), CBF-3 (Be), and ICE1-3 (Bf) plants. (C) Survival rate of plants from (A) and (B) after re-watering. (D) Survival rate of plants from three individual transgenic lines of each vector. (E) Survival rate of plants from all transgenic lines as described in methods and materials. SD (error bars) was calculated from results of three independent experiments (n=30 for each experiment). Asterisks indicate significant differences from the corresponding WT values as determined by Student’s t-tests (*0.01 P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). Experiments were repeated at least three times with similar results.

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Increase of Plant Drought Tolerance through Gene Modification
PCR products were sequenced to ensure that they encoded the expected gene products.

The pGPTV-SdaI vector was used to construct a binary expression vector. The pRNAi-vector was used to construct an intron-spliced hairpin RNA (RNAi construct), and the isolated gene or gene fragment was constructed into the relevant vector to yield pGPTV-CBF1-CBF3 (CBF), pGPTV-ICE1 (ICE1), pGPTV-eski1 (esk1), pGPTV-esk2i (esk2i), pGPTV-esk2i-CBF1-CBF3 (esk2i-CBF), pGPTV-eski1-ICE1 (esk1-ICE1), and pGPTV-esk2i-ICE1 (esk2i-ICE1). The transcription of each gene or gene fragment was under the control of the 35S promoter and 35S terminator.

Agrobacterium-mediated gene transformation

The floral dip method [44] was applied to stably transform the Arabidopsis plant by using the Agrobacterium strain ATHV containing the designed binary vector. Seeds from treated plants were germinated on media containing 1/2 MS salts, 1% sucrose, 0.8% agar and 50 μg/mL kanamycin. Resistant seedlings were transferred to soil and grown under 16 h light/8 h dark at 24°C in a growth chamber. After PCR confirmation, positive seedlings were used to produce T2 and T3 seeds, which were always subjected to kanamycin selection and PCR confirmation; only the positive plants were used for seed production of the next generation. The kanamycin-tolerant and PCR-positive T3 or T4 plants were used in all experiments. Ecotype Col-0 served as control.

Quantitative real-time PCR (qRT-PCR)

Total RNA from different Arabidopsis plants was isolated using the RNeasy plant mini kit (Qiagen, Germany) according to the manufacturer’s instructions. To eliminate any residual genomic DNA, total RNA was treated with ribonuclease-free DNase I (Thermo Scientific). Two micrograms of the total RNA was used as template to synthesize cDNA employing the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time PCR was performed according to Kant et al. [45]. The PCR reaction was performed with three replicates and repeated with three biological samples. Relative quantification values for each target gene were calculated by the 2^(-ΔΔC(T)) method [46]. For normalizing the amount of total RNA in all Arabidopsis samples, Actin2 (AT3G18780) and beta tubulin (AT5G23860) were used as internal reference genes to compare data from different PCR runs or cDNA samples. The GenScript online tool was used to design the primers (https://www.genscript.com/), and primers used in q-PCR are listed in Supplemental Table S1.
In vitro assay for plant response to ABA and PEG

For germination, seeds were planted on the plate containing 1/2 MS salts, 1% sucrose, and 0.8% agar with 0 or 0.5 μM ABA as indicated. Plates were chilled at 4°C in the dark for 3 d and moved to 24°C with a 16 h light/8 h dark cycle. The percentage of seed germination was scored at the indicated times. Germination was defined as an obvious emergence of the radicle through the seed coat [47].

To study the inhibition effect of ABA in post-germinative growth, seeds were sown on 1/2 MS medium with 30 μg/mL kanamycin for 4 d after 3 d stratification, then transferred to 1/2 MS medium containing 0 or 20 μM ABA.

For response to in vitro dehydration, 3-day-old seedlings were transferred to 1/2 MS medium previously infused with 30% PEG8000 for 14 d [48]. After growing for 14 d on the treatment medium vertically, seedlings were photographed with a digital camera.

Greenhouse test for drought tolerance

15 ESKi transgenic lines, 14 eski-ICE1 transgenic lines, 3 eski-CBF transgenic lines, 5 CBF transgenic lines and 5 ICE1 transgenic lines were used in both PEG and ABA in vitro assay. For each treatment, 30 plants of each line have been subjected to the treatment. Each test has been repeated three times. The influence of the tested element was concluded based the result pooled together from three repeats.

Measurement of drought effect on seed biomass

For germination, seeds were planted on the plate containing 1/2 MS medium with 0 or 20 μM ABA. After growing for 14 d on the treatment medium vertically, seedlings were photographed with a digital camera.

Supporting Information

Table S1 Primers used in molecular analyses.

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Author Contributions

Conceived and designed the experiments: HW FX. Performed the experiments: FX TB HX. Analyzed the data: FX HW ZL JZ JZ JK. Contributed reagents/materials/analysis tools: FX TB. Wrote the manuscript: HW FX.
promoter elements and a cold-regulatory circuit that is desensitized by low temperature. Plant Physiol 133: 910–918.

17. Chinnusamy V, Ohto M, Kannan S, Lee BH, Hong X, et al. (2003) ICE1: a regulator of cold-induced transcription and freezing tolerance in Arabidopsis. Genes Dev 17: 1045–1054.

18. Chinnusamy V, Zhu J, Zhu JK (2007) Cold stress regulation of gene expression in plants. Trends Plant Sci 12: 444–451.

19. Jaglo-Ottesen K, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Science 280: 104–106.

20. Haake V, Cook D, Riechmann JL, Pinöca O, Thomashow MF, et al. (2002) Transcription factor CBF4 is a regulator of drought adaptation in Arabidopsis. Plant Physiol 130: 639–648.

21. Siddiqua M, Nassuth A (2011) Vitis CBF1 and Vitis CBF4 differ in their effect on Arabidopsis abiotic stress tolerance, development and gene expression. Plant Cell Environ 34: 1745–1759.

22. Orabya H, Ahmad R (2012) Physiological and biochemical changes of CBF3 transgenic oat in response to salinity stress. Plant Sci 185: 186–198.

23. Zhang L, Li Z, Li J, Wang A (2013) Ectopic overexpression of SsCBF1, a CRT/ DRE-binding factor from the nightshade plant Solanum lycopersicoides, confers freezing and salt tolerance in transgenic Arabidopsis. PloS One 8: e61110.

24. Boyer JS (1982) Plant productivity and environment. Science 218: 443–446.

25. Xin Z, Browse J (1998) Eskimo1 mutants of Arabidopsis are constitutively freezing tolerant. Proc Natl Acad Sci U S A 95: 7799–7804.

26. Xin Z, Mandol Kok A, Chen J, Last RL, Browse J (2007) Arabidopsis ESK1 encodes a novel regulator of freezing tolerance. Plant J 49: 786–799.

27. Bouchabke-Coussa O, Quashie ML, Seoane-Redondo J, Fortabat MN, Gery C, et al. (2008) ESKIMO1 is a key gene involved in water economy as well as cold acclimation and salt tolerance. BMC Plant Biol 8: 125.

28. Lefebvre V, Fortat MN, Ducamp A, North HM, Maia-Grondard A, et al. (2011) ESKIMO1 disruption in Arabidopsis alters vascular tissue and impairs water transport. PloS One 6: e16645.

29. Zhang M, Barg R, Yin M, Gueta-Dahan Y, Leikin-Frenkel A, et al. (2005) Involvement of the Arabidopsis gene ᵇγ-tubulin3A in cell elongation. Plant Mol Bio 68: 533–555.

30. Duan J, Cai W (2012) OsLEA3-2, an abiotic stress induced gene of rice plays a key role in stress tolerance in Arabidopsis and grasses. Plant Physiol 149: 98–95.

31. Badawi GH, Kawano N, Yamauchi Y, Shimada E, Sasaki R, et al. (2004) Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in Zea mays L. Plant Cell Physiol 45: 1042–1052.

32. Hsieh TH, Lee JT, Chiang YY, Chan MF (2002) Tomato plants ectopically expressing Arabidopsis CBF1 show enhanced resistance to water deficit stress. Plant Physiol 130: 618–626.

33. Morran S, Eini O, Pyooverreko T, Parent B, Singh R, et al. (2011) Improvement of stress resistance of wheat and barley by modulation of expression of DREB/CBF factors. Plant Biotechnol J 9: 230–249.

34. Qin F, Sakuma Y, Li J, Liu Q, Li YQ, et al. (2006) Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in Zea mays L. PLoS One 1: e45117.

35. Qin F, Sakuma Y, Li J, Liu Q, Li YQ, et al. (2006) Cloning and functional analysis of a novel DREB1/CBF transcription factor involved in cold-responsive gene expression in Zea mays L. PLoS One 1: e45117.

36. Dong X, Gao Y, Wang Y, Jia X, Zhang Y, et al. (2009) Overexpression of a novel CBF gene in rice, Oryza sativa L., encodes transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant J 33: 753–763.

37. Morran S, Eini O, Pyooverreko T, Parent B, Singh R, et al. (2011) Improvement of stress resistance of wheat and barley by modulation of expression of DREB/CBF factors. Plant Biotechnol J 9: 230–249.

38. Xiao BZ, Chen X, Xiang CB, Tang N, Zhang QF, et al. (2009) Evaluation of seven function-known candidate genes for their effects on improving drought resistance of transgenic rice under field conditions. Mol Plant 2: 73–83.

39. Gutha LR, Reddy AR (2008) Rice DREB1B promoter shows distinct stress-specific responses, and the overexpression of cDNA in tobacco confers improved abiotic and biotic stress tolerance. Plant Mol Bio 68: 333–355.

40. Halpin C (2005) Gene stacking in transgenic plants: the challenge for 21st century plant biotechnol. Plant Biotechnol J 3: 141–155.

41. Ramana Rao MV, Parameswari C, Sripriya R, Veluthambi K (2011) Transgene stacking and marker elimination in transgenic rice by sequential Agrobacterium-mediated co-transformation with the same selectable marker gene. Plant Cell Rep 30: 1241–1252.

42. Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, et al. (2008) OsmiRE1 disrupts Arabidopsis CBF1 and CBF3 transcription factors and reduces chilling-sensitive plant growth. Proc Natl Acad Sci U S A 105: 17422–17427.

43. Yoneyama T, Kameda H, Takamura K, Itoh M, Takamiya M, et al. (2006) Nature of OsDREB genes in rice, Oryza sativa L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant J 33: 753–763.

44. Novillo F, Alonso JM, Ecker JR, Salinas J (2004) CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in Arabidopsis. Proc Natl Acad Sci U S A 101: 3985–3990.