Activated Expression of an Arabidopsis HD-START Protein Confers Drought Tolerance with Improved Root System and Reduced Stomatal Density

Hong Yu,6,1 Xi Chen,6,1 Yuan-Yuan Hong,6,1 Yao Wang,6 Ping Xu,6 Sheng-Dong Ke,6 Hai-Yan Liu,6 Jian-Kang Zhu,7 David J. Olive,6 and Cheng-Bin Xiang6,2

6 School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, People’s Republic of China
7 Institute for Integrative Genome Biology, Department of Botany and Plant Sciences, University of California, Riverside, California 92521

Drought is one of the most serious problems for sustainable agriculture worldwide. For agricultural and environmental sustainability, it is important to breed or genetically engineer crops with improved stress tolerance. The bottleneck for this endeavor has been and continues to be the identification of key genes that can be used as drought resistance markers for breeding and/or can be used directly for engineering transgenic crops with improved drought tolerance without compromising yield or biomass.

The adaptive responses to drought must be coordinated at the molecular, cellular, and whole-plant levels. It is generally believed that roots first perceive a dehydration stress signal when the water deficit reaches a certain level (Comstock, 2002). How the physical signals of dehydration stress are perceived by the roots and converted into biochemical signals still remains unclear. Abscisic acid (ABA) is involved in coordinating whole-plant responses. It is thought to be synthesized in the roots and translocated to the aerial portion of the plant, where it regulates stomatal behavior (Sauter et al., 2001). Recent studies of active pools of ABA in roots and shoots raised new questions about the long-distance signaling of ABA. In vivo imaging studies suggest that water stress applied to the root system resulted in the generation of ABA pools in the shoot but not in the root (Christmann et al., 2005). The long-sought ABA receptors remained elusive until recently. Three ABA receptors have been reported thus far (Razem et al., 2006; Shen et al., 2006; Liu et al., 2007).

Due to the complex nature of the response to drought stress, the molecular mechanisms underlying plant tolerance to drought stress and high levels of superoxide dismutase. Molecular genetic analysis and recapitulation experiments showed that the enhanced drought tolerance is caused by the activated expression of a T-DNA tagged gene that encodes a putative homeodomain-START transcription factor. Moreover, overexpressing the cDNA of the transcription factor in transgenic tobacco also conferred drought tolerance associated with improved root architecture and reduced leaf stomatal density. Therefore, we have revealed functions of the homeodomain-START factor that were gained upon altering its expression pattern by activation tagging and provide a key regulator that may be used to improve drought tolerance in plants.

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Transpirational water loss through the stomata is a key determinant of drought tolerance (Xiong et al., 2002). Regulation of stomatal behavior has been an active area of research for drought stress. Stomatal movement is a response to environmental changes and is controlled by guard cell turgor (Schroeder et al., 2001). Guard cell turgor change is influenced by many factors, such as light, phytohormones, potassium ion, calcium ion, malate, NO, and H2O2 (Assmann and Wang, 2001; Schroeder et al., 2001; Assmann, 2003; Nilson and Assmann, 2007; Shimazaki et al., 2007). Recently, a guard cell anion channel affecting guard cell turgor was identified (Vahisalu et al., 2008). Guard cell signaling is considered as a scale-free network rather than a stand-alone pathway. This scale-free network is robust and allows stomatal movement to adjust rapidly to changing environments (Hetherington and Woodward, 2003).

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stress are still not fully understood. Efforts to enhance plant stress tolerance through conventional breeding and genetic engineering have had limited success. It is anticipated that through functional genomics and comparative genomics studies, novel genes will be discovered and used to improve stress tolerance. The study of stress-responsive transcription factors has been one of the foci in the studies on drought stress tolerance. Major stress-responsive transcription factors have been extensively analyzed in Arabidopsis thaliana (Stockinger et al., 1997; Zhang et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 2006; Yamasaki et al., 2008). A subset of stress-responsive genes is under control of the CBF/DREB (for C-repeat binding factor/dehydration responsive element binding protein) family of transcription factors (Stockinger et al., 1997; Liu et al., 1998). Ectopic expression of the normally stress-induced CBF/DREB genes in transgenic plants led to constitutive expression of their downstream stress-responsive genes and improved tolerance to drought, salinity, and freezing stress (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000). Recently, an Arabidopsis NFYB (for B subunit of nuclear factor Y) family transcription factor was reported to confer drought tolerance in Arabidopsis and maize (Zea mays), implying a previously unknown drought tolerance mechanism (Nelson et al., 2007). In addition, overexpression of a rice (Oryza sativa) NAC-type transcription factor was reported to confer drought tolerance in rice (Hu et al., 2006). No yield penalty was reported for the overexpression factor was reported to confer drought tolerance in rice (Yamasaki et al., 2006; Ma et al., 2007; Shinozaki and Yamaguchi-Shinozaki, 2006). The activation of HDG11 expression enhances multiple characteristics related to drought tolerance, including enhanced root growth and reduced stomatal density. This study demonstrates the utility of a novel transcription factor gene in dramatically improving drought tolerance in Arabidopsis and tobacco (Nicotiana tabacum) and suggests that many drought tolerance effector genes are not normally under the control of this transcription factor since the deletion of the factor has no effect. These genes may come under its regulatory control if it is overexpressed. In addition, molecular biological analysis revealed major molecular mechanisms underlying the drought tolerance phenotype of the mutant.

RESULTS

Isolation of the enhanced drought tolerance1 Mutant with Improved Drought Tolerance

Here, we report the identification of a novel drought tolerance gene in Arabidopsis, HDG11, via activation tagging. HDG11 encodes a protein in the homeodomain (HD)-START transcription factor family also known as the Class IV Homeodomain-Leucine Zipper transcription factor family (Nakamura et al., 2006). The activation of HDG11 expression enhances multiple characteristics related to drought tolerance, including enhanced root growth and reduced stomatal density. This study demonstrates the utility of a novel transcription factor gene in dramatically improving drought tolerance in Arabidopsis and tobacco (Nicotiana tabacum) and suggests that many drought tolerance effector genes are not normally under the control of this transcription factor since the deletion of the factor has no effect. These genes may come under its regulatory control if it is overexpressed. In addition, molecular biological analysis revealed major molecular mechanisms underlying the drought tolerance phenotype of the mutant.
edt1 Has Altered Root Architecture with Deeper Roots and More Lateral Roots

The primary root of the mutant seedlings elongated faster than the wild type on agar medium (Figures 2A, 2B, and 2E). After growing on the medium for 3 d, the length of primary roots of edt1 was more than twice that of the wild type (Figures 2A and 2E). The mutant seedlings growing on medium also had more lateral roots at 20 d than the wild type (Figure 2C), and a markedly increased primary root length and root biomass was also observed in plants...
grown in soil under short-day conditions (Figure 2D). The mutant had more than twice as many lateral roots than the wild type from day 10 to day 20 (Figure 2F); consequently, the root dry weight of medium-grown mutant seedlings was significantly higher than that of the wild type (Figure 2G). The taproot of the mutant grown in soil was approximately twice as long as that of wild-type plants, and the root biomass of the mutant was more than twofold that of the wild type (Figure 2H). Root systems with deep roots increase the water accessibility of the plant. The altered root architecture of the mutant with longer primary roots and more lateral roots would enhance the uptake of water and nutrients, positively contributing to drought tolerance.

Reduced Leaf Stomatal Density and Increased Water Use Efficiency in the Mutant

The average stomatal density of the edf1 mutant was 30.5% less than that of the wild type (Figures 3A and 3B). The reduced stomatal density of the mutant was mainly caused by the enlarged size of epidermal cells, which had a significant reduction in cell density (of 36.1%) relative to the wild type (Figure 3B). While the stomatal density was reduced, the stomatal size was increased in the mutant (Figure 3C). The average stomatal dimension was 24.3 by 14.4 μm for the mutant in contrast with 19.0 by 12.8 μm for the wild type. The reduced stomatal density...
probably contributes to the reduced rate of water loss from \textit{edt1} leaves.

Reduced stomatal density is known to affect water and CO$_2$ exchange (Hetherington and Woodward, 2003). We thus measured photosynthesis and transpiration rates of both the wild type and the mutant and found that the rate of photosynthesis is higher in the mutant, while the rate of transpiration is lower (Figures 3D and 3E). Consequently, the water use efficiency (WUE) is higher for the mutant (Figure 3F). The increased photosynthesis rate of the mutant is unexpected, as reduced stomatal density is generally believed to decrease CO$_2$ exchange. This implies that the mutant has an enhanced photosynthesis efficiency.

Accumulation of ABA and Pro Is Increased in the \textit{edt1} Mutant in Response to Stress Treatments

ABA plays crucial roles in plant stress responses, especially during drought stress. To determine whether ABA metabolism is changed in the \textit{edt1} mutant, we used an ELISA to quantify the ABA content of the mutant and the wild type grown under the same conditions and treatments. Figure 4A shows that under normal conditions, the ABA content was higher in the mutant than in the wild type, but not significantly so. Upon exposure to 10% polyethylene glycol (PEG) 6000, a stress treatment commonly used to mimic drought tolerance in the laboratory, the ABA content became significantly higher in the mutant than in the wild type. These results suggest that input stress signals are required for the higher ABA accumulation in the mutant and that the \textit{edt1} mutation may be involved in stress signaling. The increased ABA content in the mutant under osmotic stress is consistent with its reduced water loss rate (Figure 1I).

Accompanying the ABA increase, the Pro content of the \textit{edt1} mutant was threefold that of the wild type under normal conditions and more than twice that of the wild type upon PEG treatment (Figure 4B). The elevated Pro level in the mutant is likely another important factor in enabling the \textit{edt1} mutant to cope with drought stress.

Oxidative Stress Tolerance Is Enhanced in the \textit{edt1} Mutant with Increased Superoxide Dismutase Activity

Drought stress leads to the accumulation of reactive oxygen species that need to be detoxified in order for plants to achieve drought tolerance (Chaves and Oliveira, 2004; Wang et al., 2005). To test whether the enhanced drought tolerance in the mutant might involve alterations in oxidative stress responses, we compared the responses of the mutant and wild-type seedlings to treatment with the herbicide paraquat. At 0.2 μM, paraquat caused complete bleaching of the wild type but not of the mutant plants (Figure 4C), and the survival rate of the mutant seedlings
Figure 4. Quantification of ABA, Pro, and SOD Activity in the edt1 Mutant, 35S-HDG11, and Wild-Type Seedlings.

(A) ABA contents. Ten-day-old seedlings of the wild type, edt1 mutant, and 35S-HDG11 transgenic lines were used for ABA quantification. ABA was determined by ELISA. Values are mean ± SE (n = 3 experiments, * P < 0.05, **P < 0.01). FW, fresh weight.

(B) Pro contents. Ten-day-old seedlings of the wild type, edt1 mutant, and 35S-HDG11 transgenic lines were used for Pro quantification. Pro content was measured spectrophotometrically. Values are mean ± SE (n = 3 experiments, * P < 0.05, **P < 0.01).

(C) Comparison of oxidative stress tolerance between the wild-type and edt1 mutant seedlings. The wild-type and mutant seeds were first germinated on MS medium and then transferred to MS medium containing 0 (Control) or 0.2 μM paraquat (Paraquat) and incubated under continuous light at 22°C for 1 week before the photographs were taken.

(D) Survival rate of the wild type and mutant on medium containing 0 (Control) or 0.2 μM paraquat (Paraquat) over a period of 1 week. Survival rate (y axis) was defined as the percentage of the wild-type control. Values are mean ± SE (n = 50 plants, **P < 0.001).

(E) Comparison of SOD activity between the edt1 mutant and the wild type. Ten-day-old mutant and wild-type seedlings were treated with 0 or 10% PEG 6000 in liquid MS medium for 6 h, and then SOD activity was assayed and presented as folds of the wild-type control. Values are mean ± SE (n = 3 experiments, * P < 0.05, **P < 0.01).

(F) Estimation of SOD mRNA level in the edt1 mutant and the wild type by RT-PCR. Total RNA was isolated from 1- and 2-week-old seedlings (1W and 2W) and leaves (L) and roots (R) of 4-week-old plants grown in soil. SOD transcript levels were estimated by RT-PCR for 30 cycles with specific primers for SOD. Tubulin (Tub) transcript levels serve as an equal loading standard. The experiment was repeated three times, and the typical result of an ethidium bromide–stained agarose gel is presented.

(G) Estimation of SOD mRNA level by real-time RT-PCR. Using the same sample and primers as in (F), real-time RT-PCR was performed for 30 cycles. The relative transcript level was obtained as folds of the tubulin transcript level, which was used as the internal control. Values are mean ± SE (n = 3 experiments).
was significantly higher than that of the wild type (Figure 4D), indicating that the mutant was substantially more tolerant to oxidative stress.

Superoxide dismutases (SODs) are important antioxidant enzymes that detoxify superoxide free radicals (Van Camp et al., 1990; Arisi et al., 1998; Kliebenstein et al., 1998). The edt1 mutant showed higher tolerance to paraquat than the wild type, indicating an enhanced capability to scavenge reactive oxygen species. Consistent with this notion, SOD activity assays showed a significantly higher activity in the edt1 mutant than in the wild type under both control and PEG-stressed conditions (Figure 4E). The elevated SOD activity was positively correlated with the transcript levels of a Cu/Zn SOD as revealed by RT-PCR and real-time RT-PCR analyses (Figures 4F and 4G). These results are consistent with SOD induction by ABA (Jiang and Zhang, 2002).

Activated Expression of the Tagged Gene HDG11 in the Mutant

Genetic analyses showed that the mutant phenotype cosegregated with the herbicide resistance marker gene residing on the T-DNA and was controlled by a single dominant locus (see Supplemental Table 1 online), consistent with the DNA gel blot result of a single T-DNA insert (Figure 5A). These results suggest that the enhancers residing on the T-DNA might have activated a gene(s) at the integration site of the T-DNA. Molecular analysis identified that the T-DNA integration site was in the 5′ untranslated region (UTR) of At1g73360 on chromosome one. The exact site of integration of the T-DNA right border with four 3SS enhancers was 50 bp upstream of the ATG initiation codon of At1g73360 (Figure 5B). The T-DNA insertion did not disrupt the rest of the gene, and the coding region is identical to that of the wild type. The T-DNA tagged At1g73360 locus consists of 10 exons and nine introns. The open reading frame is predicted to encode a protein of 722 amino acids with an estimated molecular mass of 79 kD with an HD near the N terminus and a START domain in the middle (Figure 5B). The At1g73360 gene was previously named HDG11 (Nakamura et al., 2006). This type of protein with a combination of HD plus START domain is only found in plant genomes. RT-PCR analysis revealed that while HDG11 was highly activated, its neighboring genes (At1g73350 and At1g73370) were not (Figure 5C). This result was confirmed with real-time RT-PCR analysis (Figure 5D).

In wild-type plants, RT-PCR analysis indicated that HDG11 expression was undetectable in vegetative tissues but that the gene was expressed at low levels in the reproductive organs (Figures 5E and 5F). The expression of the HDG11 gene was dramatically altered in the mutant, where high levels of HDG11 transcript were detected in all organs analyzed (Figures 5E and 5F). The altered expression pattern and elevated transcript levels of HDG11 were apparently caused by the T-DNA insertion. Since the four tandem repeats of the 3SS enhancers were inserted in the 5′ UTR, the regulation by the HDG11 promoter was likely abolished. Instead, the 3SS enhancers caused strong expression of HDG11 in a constitutive fashion. These results suggest that the activated expression of HDG11 caused the mutant phenotype.

Analysis of the HDG11 Protein Localization, the Response of HDG11 to Stress Treatments, and the Loss-of-Function Mutants

To localize the HDG11 protein, a gene encoding HDG11 fused to the N terminus of green fluorescent protein (GFP) was constructed. The fusion construct was delivered into onion epidermal cells. Figure 6A shows the nuclear localization of the HDG11-GFP fusion protein and thus presumably HDG11 accumulates in the nucleus.

RNA gel blot analysis was conducted to examine if HDG11 is responsive to drought and other abiotic stresses. Figure 6B shows that the HDG11 transcript is not detectable in response to PEG, ABA, and salt or oxidative stress treatment. This is consistent with the compiled microarray data on The Arabidopsis Information Resource website (www.arabidopsis.org).

To perform loss-of-function analysis, T-DNA insertion mutant lines SALK_044434, a mutant allele of At1g73380 (HDG11), and SALK_061323, a mutant allele of At1g17920 (HDG12) with the highest amino acid sequence similarity (80%) to HDG11, were obtained and their homozygous lines were identified through a genomic PCR screen (see Supplemental Figure 1 online). The single (hdg11 and hdg12) and double (hdg11 hdg12) mutants were subjected to the drought tolerance assay. Knockout lines of either or both of these genes had no apparent difference in drought sensitivity compared with the wild type (Figure 6C). Furthermore, no apparent morphological difference was observed between the wild type and the mutants throughout development, except that the double mutants developed unusually branched trichomes (Nakamura et al., 2006). These results suggest that HDG11 in the wild type plays a minor role in drought stress response if any at all.

Drought Tolerance Phenotype Can Be Recapitulated by Overexpressing HDG11 cDNA in Wild-Type Arabidopsis

To confirm that the activated expression of HDG11 caused the drought tolerance phenotype of the mutant, recapitulation experiments were conducted. We cloned HDG11 cDNA into the expression binary vector pCB2004 in which the cauliflower mosaic virus 35S promoter drives expression of the HDG11 cDNA (Figure 6D) and generated transgenic lines of Arabidopsis (Figure 6E). The HDG11 overexpressor lines (OX1, OX2, and OX3) showed significantly improved drought tolerance compared with the wild type. After 2 weeks of drought stress, the wild-type plants displayed severe wilting symptom, while the transgenic plants did not show any wilting symptom (Figure 6F). Compared with the wild-type plants, the transgenic plants also showed a slower rate of water loss from leaves (Figure 6G). ABA and Pro contents of the transgenic plants were similar to those of the edt1 mutant and were significantly higher than those of the wild type (Figures 4A and 4B). These results demonstrate that overexpressing the HDG11 cDNA in the wild type can recapitulate the drought tolerance phenotype of the gain-of-function edt1 mutant, strongly supporting the hypothesis that the drought tolerance phenotype was caused by HDG11 overexpression.
Constitutively Overexpressing HDG11 Confers Drought Tolerance in Transgenic Tobacco

The recapitulation experiments were also performed heterologously in tobacco. The same genetic construct, pCB2004-HDG11, used in the above recapitulation experiment was transferred into tobacco and transgenic lines were generated. Several overexpressors were selected for further investigation (Figure 7A). Three independent T3 homozygous lines are shown in Figure 7. The transgenic tobacco lines overexpressing HDG11 demonstrated dramatically improved drought tolerance (Figures 7B to 7E). Under the drought assay conditions, all transgenic plants survived, while none of the control plants did. The stomatal density was reduced by 40% compared with the control transformed with the empty vector pCB2004 (Figures 7F and 7G) and the stomata were larger.
WUE was significantly increased in the transgenic lines (Figure 7H). Transgenic tobacco lines also displayed altered root architecture, as observed in the edt1 Arabidopsis mutants. The longest root was significantly longer in the transgenic plants compared with the control (Figures 7J and 7K). The biomass of both roots and leaves was also significantly increased in the transgenic plants (Figure 7L). These results with transgenic tobacco further demonstrate the great potential of this gene to improve drought tolerance of crops.

Analysis of Transcript Levels of Stress-Responsive Genes and of Root- and Stomata-Related Genes

To begin to explore the molecular mechanisms underlying the HDG11 overexpression-caused changes in drought tolerance,
Figure 7. Overexpression of At HDG11 Confers Drought Tolerance in Transgenic Tobacco Plants.  

(A) The same pCB2004-HDG11 construct was used to transform tobacco. The empty vector pCB2004 was used to generate a transgenic control. Total RNA was isolated from leaf tissue of T0 lines and subjected to RNA gel blot analysis. The blot was probed with HDG11 cDNA, and RNA gel serves as loading control. The experiment was repeated three times, and a typical result of RNA gel blots is presented. The three independent lines indicated by asterisks were chosen for further analysis.  

(B) to (E) Drought tolerance assay. T2 homozygous plants of the three independent transgenic tobacco lines were assayed for drought tolerance. Transgenic and control plants were grown in identical pots with the same amount of soil (weight). The top three rows were the transgenic tobacco plants (35S-HDG11), and the bottom row was the control plants. One-month-old plants (B) were withheld watering for 10 d (C) and continued for 20 d (D). Then watering was resumed to allow plants to recover. Plants were recovered for 14 d (E). Twelve plants of each line were used for one experiment. Three replica experiments were conducted.  

(F) Images of the lower epidermis of the fourth fully expanded leaf from a 1-month-old control and a representative transgenic plant. Both images were captured at the same magnification of ×200.  

(G) Comparison of stomatal density between 1-month-old control and transgenic plants. Twenty-five to thirty microscopic sights were observed for each plant. Five plants were used for each line. Values are mean ± SE (**P < 0.001).  

(H) Comparison of stomatal size between one-month-old control and transgenic lines. Ten stomata were measured for each sight. Twenty-five to thirty microscopic sights were observed for each plant. Five plants were used for each line. Values are mean ± SE (**P < 0.001).  

(I) Comparison of WUE between 1-month-old control and transgenic plants. Three measurements were made for each plant, and five plants were used for each line. Values are mean ± SE (**P < 0.001).  

(J) Whole root system of 12-week-old control and transgenic plants.  

(K) Comparison of the longest root length. The longest root of the 12-week-old plants as in (J) was measured. Ten plants were used for each line. Values are mean ± SE (P < 0.05).  

(L) Comparison of biomass of roots and leaves. The fresh weights of roots and leaves of 12-week-old control and transgenic plants were recorded. Before weighing, roots were carefully washed to remove soil and blotted dry. Ten plants were used for each line. Values are mean ± SE (P < 0.05, **P < 0.01).
HDG11 Can Transactivate CIPK3, NCED3, and ERECTA Promoters

A search for HD binding sites in the promoter of the above HDG11-regulated genes was conducted. It was found that NCED3, LOS5/ABA3, CIPK3, CAX3, ABI3, RGAL, IAA28, and ERECTA all contain at least one HD binding site in their promoter. These genes are potentially the primary targets of HDG11; therefore, HDG11 may directly activate NCED3, LOS5/ABA3, CIPK3, CAX3, ABI3, and ERECTA as a transcription activator and downregulate RGAL and IAA28 as a transcription repressor.

To demonstrate experimentally that HDG11 acts as a transcription activator, reporter genes were constructed to include the promoter of CIPK3, NCED3, and ERECTA fused to a GFP reporter (Figure 8A). These reporter constructs were cobombarded into onion cells with either the 35S-HDG11 construct as effector or a modified 35S-HDG11 N-terminal deletion where the HD domain was removed as a negative effector control. If HDG11 can transactivate these reporter genes, GFP should be visible in the presence of the 35S-HDG11 effector but not in the negative control with the inactive effector. The results shown in Figure 8B demonstrated that HDG11 expressed by 35S-HDG11 was indeed able to activate all three of these reporter constructs, while the modified HDG11 construct activated none of the reporters. This shows that the intact HDG11 factor was able to bind to the HD sites in the promoters of the three reporter genes and activate their expression in vivo.

DISCUSSION

Drought Tolerance Phenotype of the edt1 Mutant Is Facilitated by Multiple Stress Tolerance Determinants Caused by a Single Transcription Factor

We isolated a mutant that is significantly more tolerant to drought stress than wild-type Arabidopsis. The edt1 mutant grows and develops relatively normally in unstressed conditions. The

| Table 1. Relative Expression Levels of Stress-Responsive Genes in Response to Stress Treatment |
|---------------------------------|----------------|----------------|----------------|
|                                | Control        | 30% PEG 6000   |                |
| Gene                           | Wild Type      | edt1 Mutant    | Wild Type      | edt1 Mutant    |
| RD29A                          | 1.00 ± 0.10    | 1.84 ± 0.15    | 18.77 ± 2.05   | 26.35 ± 2.29   |
| P5CS                           | 1.00 ± 0.08    | 1.40 ± 0.14    | 37.50 ± 2.17   | 150.12 ± 14.20 |
| NCED3                          | 1.00 ± 0.07    | 1.97 ± 0.19    | 40.79 ± 2.92   | 178.53 ± 14.14 |
| ABA3                           | 1.00 ± 0.05    | 5.46 ± 0.41    | 49.74 ± 3.52   | 245.51 ± 18.46 |
| CIPK3                          | 1.00 ± 0.08    | 3.16 ± 0.35    | 53.08 ± 5.00   | 79.89 ± 9.37   |
| CAX3                           | 1.00 ± 0.13    | 3.97 ± 0.44    | 5.62 ± 0.49    | 19.43 ± 1.54   |
| ABI3                           | 1.00 ± 0.09    | 2.60 ± 0.33    | 8.69 ± 1.09    | 18.64 ± 2.04   |

Seedlings were grown on half-strength MS agar media containing 20 g/L sucrose under continuous light. Ten-day-old wild-type and mutant seedlings were treated with water (control) or 30% PEG 6000 for 3 h. RNA isolation and real-time RT-PCR were performed. Relative transcript levels were normalized to the wild-type control after internal control normalization. Values are mean ± SE (n = 3 experiments).
drought tolerance phenotype of the mutant is likely the result of a collection of beneficial factors observed in the mutant.

First, the mutant phenotype is associated with improved root architecture, a more extensive root system, and reduced stomatal density, all of which contribute to improved water homeostasis (Hetherington and Woodward, 2003; Malamy, 2005; Masle et al., 2005). Second, the higher SOD activity and Pro content in the mutant contribute to better osmotic adjustment and ROS detoxification, which are important determinants of drought tolerance (Kishor et al., 1995; Chen and Dickman, 2005). Third, the higher ABA content not only contributes to the reduced water loss rate observed in mutant leaves, but more importantly may expand the capacity for stress response in the mutant. Fourth, the mutant is more responsive to stress signaling, as reflected by augmented ABA and Pro accumulation as well as the transcript levels of many stress responsive genes examined.

The ability to respond to stress signaling is beneficial to plants as it allows normal growth and development under low stress conditions, while enabling a stringent response once stress occurs. This trait would be highly desirable for crop improvement. Unlike other transcription factors, such as DREB factors (Kasuga et al., 1999), constitutive overexpression of HDG11 does not cause growth retardation.

All of these drought tolerance determinants in the edt1 mutant are caused by the ectopic overexpression of a single transcription factor, HDG11. Recapitulation experiments further confirmed that ectopic overexpression of HDG11 can confer a drought tolerance phenotype to wild-type plants. Apparently, the dramatic change of the restricted expression pattern of HDG11 generated a neomorphic mutation. The neomorphic mutation might create an unphysiological status in the mutant but showed no apparent adverse effects on growth and development. Furthermore, overexpression of HDG11 in tobacco also resulted in significantly improved drought tolerance, improved root architecture, and reduced stomatal density, suggesting great promise as a candidate gene for engineering crops with improved drought tolerance.

Another interesting observation is that the reduced stomatal density did not decrease photosynthesis, as one would expect if gas exchange were limited by a reduced number of stomata. There are two possible explanations for this. First, the increased size of stomata might have compensated for the reduced

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### Table 2. Relative Expression Levels of Mutant Phenotype-Related Genes

| Gene       | Wild-Type Shoot | edt1 Shoot | Gene       | Wild-Type Root | edt1 Root |
|------------|-----------------|------------|------------|----------------|-----------|
| ERECTA     | 1 ± 0.080       | 12.21 ± 1.620 | IAA28     | 1 ± 0.052       | 0.18 ± 0.012 |
| KAT1       | 1 ± 0.060       | 0.21 ± 0.013 | RGAL      | 1 ± 0.061       | 0.12 ± 0.011 |

Seedlings were grown on half-strength MS agar media containing 20 g/L sucrose under continuous light. RNA was isolated from shoots and roots, and real-time RT-PCR was performed. Relative transcript levels were normalized to either wild-type shoot (ERECTA and KAT1) or wild-type root (IAA28 and RGAL) after internal control normalization. Values are mean ± SE (n = 3 experiments).

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**Figure 8.** Transient Assays for the Transactivation of CIPK3, NCED3, and ERECTA Promoters by HDG11.

(A) Illustration of the reporter and effector constructs (not drawn to scale).

(B) Fluorescent images of onion cells transfected with the reporter and effector combinations as indicated via bombardment. The 35S-GFP construct served as positive control, while the 35S-RFP construct served as an internal control for every bombardment. RFP, red fluorescent protein.
stomatal density, but this should have caused more transpiration. Second, HDG11 might promote the rate of photosynthesis through some unknown mechanisms in addition to the increased SOD activity and Pro content. Enhanced antioxidation and increased Pro levels are known to increase photosynthesis efficiency (Gupta et al., 1993; De Ronde et al., 2004).

The increased leaf cell size in the mutant is similar to that caused by ERECTA overexpression (Masle et al., 2005), and ERECTA is upregulated in the mutant. It is possible that ERECTA may be involved in regulating the cell cycle, or its overexpression might affect cell size. This awaits further investigation.

Molecular Mechanisms Underlying the Drought Tolerance of the Mutant

How exactly the HDG11 gene mediates stress tolerance in the edt1 mutant is unclear. As a transcription factor, HDG11 might be expected to regulate a suite of target genes when overexpressed. Based on our experimental results, HDG11 may regulate, through direct and indirect actions, a complex network, which includes various genes that bring about increased accumulation of ABA, enhanced ABA and calcium signaling, enhanced oxidative stress tolerance, improved root architecture, and reduced stomatal density of the mutant.

Since ABI3 complexes with bZIP factor ABI5 and 14-3-3 proteins in the nucleus (Himmelbach et al., 2003), it is possible that the upregulation of ABI3 in edt1 may augment the capacity of the nuclear complex and thus potentiate the responsiveness of the mutant to stress signals. Moreover, ABI5 is subjected to posttranslational modification in response to these signals. This nuclear complex may serve as one of the integration points for input stress signals. This may explain why input stress signals are required for triggering the complete stress response in the mutant.

Why did knocking out HDG11 or HDG12 or even HDG11 and HDG12 not show any effect on drought tolerance? One explanation is that the genes regulated by HDG11 in the mutant are not normally under the control of this transcription factor because of its low expression in the wild type. This is consistent with the expression pattern of HDG11, which is neither normally expressed in the vegetative tissues of wild-type plants nor inducible by stress treatments (Figures 5 and 6). When HDG11 is constitutively overexpressed, a high level of HDG11 factor is likely to accumulate in the nucleus, making HDG11 a dominant transcription factor competing for any available HD binding sites. Under this circumstance, many genes with HD binding sites in their promoter may come under the regulatory control of the HDG11 factor. Depending on their promoter context, these genes can be either activated or repressed by the HDG11 factor. It is possible that HDG11 overrules the function of some other HD factors involved in stress responses (Soderman et al., 1999; Deng et al., 2002; Himmelbach et al., 2002; Johannesson et al., 2003; Zhu et al., 2004) and establishes different levels of gene expression and thereby a drought-resistant phenotype.

In addition to being a transcription factor, the possibility that HDG11 may play other roles cannot be ruled out. The HDG11 protein may interact with other proteins that function in drought tolerance and development. It is possible that other HD proteins interact with HDG11 through dimerization, for instance. Moreover, the START domain of the HDG11 factor possibly binds to an unidentified lipid ligand, which may play important roles in development and drought tolerance. Therefore, overexpressed HDG11 factor may compete for the ligand of the START domain and consequently perturb the signaling pathway. These possibilities await further investigation.

We have just begun to unravel the molecular mechanisms underlying drought tolerance in the edt1 mutant. Further investigation is underway to systemically dissect the major molecular mechanisms underlying drought tolerance in this mutant.

Is HDG11 Evolving from a Developmental Regulator toward a Stress Regulator?

The HD-START family of proteins has two known conserved domains: the homeobox domain and the START domain (Schrick et al., 2004). Between the homeobox and START domain is a putative Leu zipper motif that might be involved in dimerization. The homeobox domain is responsible for DNA binding. The START domain contains ~200 amino acids, and its function is well studied in animals. The steroidogenic acute regulatory protein binds cholesterol via the START domain and transports it from the outer to the inner mitochondrial membrane (Stocco, 2001; Romanowski et al., 2002; Strauss et al., 2003). It is not known whether it has similar functions in plants and what its ligands might be. The START domain–containing proteins are more abundant in plant genomes than in animal genomes. Most of the START domain–containing proteins in plants contain a homeodomain (Schrick et al., 2004).

Although HD proteins are usually developmental regulators, a few HD proteins have been reported to play important roles during abiotic stress tolerance in Arabidopsis (Soderman et al., 1999; Deng et al., 2002; Himmelbach et al., 2002; Johannesson et al., 2003; Zhu et al., 2004). These HD proteins do not contain the START domain.

HDG11 is likely involved in development during the reproductive phase in the wild type because its expression pattern is confined to flower buds, flowers, and immature siliques (Figure 5F), and its amino acid sequence shows extensive homology to the known development regulators of HD-START proteins (Nakamura et al., 2006). A recent report also documented that this gene might have redundant functions in development since knockout mutants did not show any abnormal phenotype, except for branched trichomes (Nakamura et al., 2006). In addition, the gene did not respond to stress treatments (Figure 6) and the knockout mutant had no phenotype, suggesting that HDG11 may at best play a minor role in stress responses.

Since the coding sequence of HDG11 is not altered in the edt1 mutant, the only difference between edt1 and the wild type lies in the altered expression pattern of HDG11. It is well known that mutations in regulatory sequences play a predominant role in evolution (Carroll, 2005). Our results with the edt1 mutant and recapitulation analysis confirm that it is the altered expression pattern of HDG11 by activation tagging in the mutant or by overexpression in the transgenic plants that confers the drought tolerance phenotype. These results suggest that the altered expression pattern of the HDG11 gene has allowed HDG11 to
gain novel functions in drought tolerance and are consistent with the King and Wilson hypothesis concerning the predominant role of regulatory mutations in organismal evolution (King and Wilson, 1975). From this point of view, this finding may reveal how drought tolerance is evolving, as changing the expression pattern of HDG1 may be one way that drought tolerance can evolve in nature.

METHODS

Generation of the Activation Tagging Library and Mutant Screen

An activation tagging library of ∼55,000 individual lines was generated with pSKI015 in the Columbia ecotype as described (Weigel et al., 2000). T2 seeds were collected as individual pools of ∼2000 independent lines. To screen for drought-tolerant mutants, T2 plants were first selected for herbicide resistance. The resistant plants were transferred to soil pots and grown side by side with the same age wild-type plants in the greenhouse. When plants were ∼2 weeks old, watering was withheld until severe shoot wilting occurred. Mutants with improved drought tolerance relative to wild-type plants were visually identified and rescued for further analysis.

Morphological Characterization of the Mutant Roots and Shoots

The edt1 mutant was characterized for morphological changes under long-day (16-h light/8-h dark) and short-day (12-h light/12-h dark) photoperiods in a growth chamber with a constant temperature of 22°C. Root morphology was examined on half-strength MS medium solidified with 1.2% agar. Briefly, mutant seeds were germinated on half-strength MS medium containing 50 mg/L glufosinate ammonium, and five herbicide-resistant seedlings and five wild-type seedlings were transferred to one half-strength MS medium plate and grown vertically for 3 d before measurement of primary root length. Four replicate plates were used. The seedlings were allowed to grow for 2 weeks before lateral roots were counted. The root biomass was dried and weighed. Ten wild-type and mutant plants were grown in soil under the same conditions in the greenhouse, and the root biomass was measured as fresh weight.

Measurements of Leaf Stomatal Density, Photosynthesis Rate, Transpiration Rate, and WUE

To measure stomatal density, leaves of the same age and from the same relative position were sampled from rosette stage plants of the wild type and mutant grown under the same short-day conditions. A leaf surface imprint method was used. Briefly, a drop of crazy glue was applied to a glass slide, and the adaxial side of a sampled leaf was pressed on the glue for ∼30 s. The leaf was removed and the imprint on the glass slide was observed under a light microscope. For statistical analysis of stomatal density, five leaves were sampled for each plant and five plants were sampled for the wild type and the mutant. Photosynthesis (P) and transpiration (T) rates were measured using a portable photosynthesis system (LI-COR LI-6400) in the morning (10:00 to 11:00 AM) under natural sunlight on the same plants mentioned above before stomatal observation. Five measurements were made for each plant, and five plants were used for the wild type and the mutant. WUE was defined as P/T ratio and derived from the measured P and T.

Drought Tolerance Assays

Drought tolerance assays were performed for both seedlings and 4-week-old plants. For seedlings, mutant and wild type seeds were separately germinated at high density in soil in 2 × 2-inch pots. The mutant seedlings were selected for herbicide resistance by spraying 0.2% commercial glufosinate ammonium when cotyledons were open. When seedlings were 10 d old, watering was withheld for 15 d before rewatering. To test drought tolerance more vigorously at later developmental stages, one mutant plant was grown with one wild type in the same 5 × 5-cm pot under short-day conditions in the greenhouse. When plants were 4 weeks old, watering was withheld for 2 weeks.

Water loss rates were measured using 10 plants each for the wild type and the edt1 mutant. Four-week-old plants were detached from their roots and weighed immediately. Then, the plant was placed in a plate on a laboratory bench and weighed at designated time intervals. The proportion of fresh weight lost was calculated on the basis of the initial weight of the plant.

Oxidative Stress Tolerance Assay

Ten-day-old seedlings of the edt1 mutant and wild type grown on half-strength MS agar plates were transferred into half-strength MS liquid medium supplemented 10% PEG 6000 and incubated at 22°C under continuous light for 2 d. One gram of the seedlings was used for ABA quantification by the ABA immunoassay kit as described (Yang et al., 2001).

Pro Measurement

Ten-day-old seedlings of the edt1 mutant and wild type grown on half-strength MS agar medium containing 2% sucrose were transferred into half-strength MS liquid medium supplemented with 10% PEG 6000 and incubated at 22°C under continuous light for 2 d. Pro concentration was determined as described (Bates, 1973).

SOD Activity Assay

SOD activity was determined according to the method previously described (Hodges and Forney, 2000). The reaction mixture includes 65.0 mM potassium phosphate, pH 7.5, 0.01 mM EDTA, 0.5 mM xanthine, 0.13 mM cytochrome C, and 0.025 units xanthine oxidase. SOD activity was determined by monitoring the inhibition of the reduction rate of cytochrome C between the reaction mixture and the control without protein extract (up to 200 μg protein) at 500 nm.

DNA and RNA Gel Blot Analysis

Genomic DNA gel blot analysis for the edt1 mutant and the wild type was performed as described (Xiang et al., 1997). RNA gel blot analysis was performed as described (Xiang and Oliver, 1998).

Identification of the T-DNA Tagged Locus

The single T-DNA insertion site in the edt1 mutant was cloned by the plasmid rescue method (Weigel et al., 2000) and positively identified by sequencing.

Genetic Analysis

Cosegregation analysis of drought tolerance and T-DNA was performed for the T2 segregating population. T2 plants were grown in 5 × 5-cm2 pots.
(one plant per pot) in a greenhouse under the short-day conditions described above. When plants were ~4 weeks old, the drought tolerance assay was conducted as described above. Meanwhile, the genotype for T-DNA insertion was identified for each plant by genomic PCR using HDG11-specific primers (see Supplemental Table 2 online) and a T-DNA right-border primer (Weigel et al., 2000). The PCR results were further confirmed by herbicide resistance by a leaf paint method whereby 0.2% commercial glufosinate ammonium ammonium herbicide was painted on an ~1-cm² area on one rosette leaf. The painted area stays green if the plant is resistant to the herbicide and becomes yellow in the wild type.

Wild-type Columbia as the female parent and the edt1 mutant as the pollen donor were backcrossed. The F1 population was tested for drought tolerance as described above, and herbicide resistance was scored by the leaf paint method.

Recapitulation Analysis

The HDG11 cDNA was isolated from the mutant by RT-PCR, cloned into pDONR207, and subsequently shuttled into the expression binary vector pCB2004 (Lei et al., 2007). The construct was introduced into Agrobacterium tumefaciens CS8C1, which was used to transform the Columbia wild type as described (Clough and Bent, 1998). Drought assays were performed on T2 transgenic plants harboring an overexpression construct to reconfirm the HDG11 gene function.

Overexpression of At HDG11 in Tobacco for Drought Tolerance Assay

To generate transgenic tobacco, the above-mentioned pCB2004-HDG11 construct was used to transform tobacco as described (Horsch et al., 1985). Primary transformants that tested positive by DNA and RNA gel blot analyses were further analyzed. Drought tolerance assays were performed on T2 transgenic lines and the control (the wild type) using the leaf paint method.

RT-PCR Analysis

Total RNA was prepared from tissues indicated in the figures by the TRIZOL reagent (Invitrogen), and 1 μg of RNA from each sample was used for the reverse transcription reaction. Subsequently, 1 μL of the reverse transcription reaction was used as template for PCR amplification. The PCR products were examined on a 0.8% agarose gel stained with ethidium bromide. The same RNA samples and primers were used for real-time PCR analysis that was performed and statistically analyzed as described (Livak and Schmittgen, 2001). SYBR green was used to monitor the kinetics of PCR product in real-time RT-PCR. As an internal control, the tubulin transcript was used to quantify the relative transcript level of each target gene in each tissue type. The primer pairs are listed in Supplemental Table 2 online. The number of PCR cycles was 30 unless otherwise specified. Three biological experiments were conducted.

Genomic PCR Screen for hdg11 and hdg12 Knockout Mutants

T-DNA insertion lines SALK_044434 and SALK_061323 were obtained from the ABRC and screened for homozygous progeny as described using specific primers and the T-DNA primer suggested (Alonso et al., 2003). The hdg11 hdg12 knockout double mutant was provided by Taku Takahashi (Nakamura et al., 2006).

Localization of the HDG11-GFP Fusion Protein

The HDG11 cDNA coding region was cloned into the binary vector pCB2008E (Lei et al., 2007) so that HDG11 sequence was in front of and in frame with GFP. The construct was subsequently delivered into onion epidermal cells by microprojectile bombardment using a PDS-1000/He biolistic particle 18 delivery system (Dupont), essentially according to the manufacturer’s instructions. Onion epidermal cell layers were placed on 1% agar plates with half-strength MS salts and bombarded using a rupture disk of 900 Pascal per square inch at a target distance of ~10 cm. At 24 h after bombardment, GFP fluorescence was analyzed with the 488-nm argon laser 19 using a Zeiss Axioplan microscope (Carl Zeiss). Images from the microscope were captured with a video camera (AxioCam HRC) connected to a computer with AxiosVision 3.0.6.1 as described (Ohkama-Ohtsu et al., 2007).

Transient Transactivation Assay

The promoters of CIPK3, NCED3, and ERECTA were amplified and cloned into the binary vector pCB2008E (Lei et al., 2007). The effector construct 35-HDG11 was the same as for the recapitulation analysis. An HDG11 N-terminal deletion without the HD domain was constructed in the same vector pCB2004 as for the effector construct and used as a negative control. The construct combinations as indicated were delivered into the lower epidermal layer of onion via microprojectile bombardment, and fluorescence was analyzed as described above.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: HDG11, AT1g73360; HDG12, AT1g71920; Tubulin, AT5G23860; Cu/Zn SOD, AT1G08830; PSCS, AT2G39800; NCED3, AT3G14440; RD29A, AT5G52310; ABI3, AT3G24650; CIPK3, AT2G26980; CAX3, AT3G51860; ABA3, AT1G16540; KAT1, AT5G46240; RGAL, AT1G66350; IA28, AT5G25890; ERECTA, AT2G26330.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Screen for hdg11 and hdg12 Knockout Mutants.

Supplemental Table 1. Genetic Analysis of Drought Tolerance in the edt1 Mutant and Cosegregation Analysis of Drought Tolerance with Herbicide Resistance.

Supplemental Table 2. PCR Primers Used in This Study.

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Activated Expression of an *Arabidopsis* HD-START Protein Confers Drought Tolerance with Improved Root System and Reduced Stomatal Density

Hong Yu, Xi Chen, Yuan-Yuan Hong, Yao Wang, Ping Xu, Sheng-Dong Ke, Hai-Yan Liu, Jian-Kang Zhu, David J. Olive and Cheng-Bin Xiang

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